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# REGULATION OF FUNCTIONAL MUSCLE TRANSFER OUTCOME BY MEANS OF AN INTERPOSED NERVE GRAFT

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## ABSTRACT: REGULATION OF FUNCTIONAL MUSCLE TRANSFER OUTCOME BY MEANS OF AN INTERPOSED NERVE GRAFT

Facial palsy is a devastating condition. The gold standard treatment for chronic unilateral facial palsy is a functional muscle transfer reinnervated by a branch of the contralateral facial nerve via a cross-facial nerve graft. However, the few published long-term clinical series indicate that the outcome is unpredictable. Approximately half of all patients achieve a clinically satisfactory result, the rest experiencing underactivity or overactivity of the transferred muscle.

Previous work using an animal model of functional muscle transfer has established that altering donor motor input to heterotopically transferred muscle correlates with reinnervation and force production, the corollary being that standardising donor motor input should standardise outcome. However there is presently no way of delivering a consistent motor input to a functional muscle transfer. The interposed nerve graft has been identified as potentially able to regulate motor input reaching a functional muscle transfer, ultimately controlling force production and clinical outcome. This project tests the hypothesis in an animal model that an interposed nerve graft of low axon regenerate transmitting capacity can standardise the outcome of functional muscle transfers despite variability in motor input, by comparing reinnervation of and force production by functional muscle transfers reinnervated with different sized motor inputs via high and low capacity interposed nerve grafts.

Force production is measured in vivo after recovery of transplanted muscle, and reinnervation is measured by nerve morphometry of both the interposed nerve graft and the neural pedicle to transplanted muscle. In support of the study hypothesis, both reinnervation and force production data suggest that outcome correlates with donor nerve magnitude only when a high capacity interposed nerve graft is used, this correlation being lost when using a low capacity interposed nerve graft. The implications for human facial reanimation surgery are discussed.

## Declaration

I declare that the work presented in this thesis is my own original work, and is that upon which I expect to be examined.

Fulvio Urso-Baiarda

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I would like to thank the many people who have contributed to this work through their support, kindness and friendship. Immeasurable thanks belongs to my parents and brother, whose lifelong love and support have been matched only by their pride for my achievements. My mother will be missed very dearly.

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*This work is dedicated to my darling Rebecca, whose patience and never-ending support continued to encourage me in this, as in all things.*

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## Abbreviations

| | |
|---|---|
| ABC | Avidin-Biotin Complex |
| CFNG | Cross Facial Nerve Graft |
| CSA | Catalyzed Signal Amplification |
| DAB | DiAminoBenzidine |
| DDSA | DoDecenyl Succinic Anhydrate |
| DPX | Distyreue-Plasticizer-Xylene |
| EDTA | EthyleneDiamineTetraAcetic acid |
| FMT | Functional Muscle Transfer |
| IMS | Industrial Methylated Spirits |
| MM | Marginal Mandibular branch of facial nerve (smaller donor nerve branch) |
| MNA | Methyl Nadic Anhydrate |
| PIPES | PIPerazine-N-N'-bis(2-Ethane Sulphonic acid) |
| S | Saphenous nerve (larger nerve graft) |
| SB | medial Saphenous nerve Branch (smaller nerve graft) |
| SD | Standard Deviation |
| SPSS | Statistics Package for the Social Sciences |
| TBS+T | Tris Buffered Saline plus Tween buffer |
| VRB | Ventral Ramus of Buccal branch of facial nerve (larger donor nerve branch) |

## Figures & Tables

# CHAPTER 1

# INTRODUCTION

## 1.1. Impact of Facial Palsy

There are aesthetic, functional and psychological consequences of facial palsy. Aesthetic consequences are reduced facial muscular tone, drooping of facial features and, in the case of one-sided facial palsy, asymmetry at rest and exaggerated facial contortions to the functional side. Functional problems relate to inadequate eye closure with risk of exposure keratitis, drooling secondary to oral incontinence, speech problems and swallowing difficulties. The face is a focal point for social interactions and affected patients may suffer psychological consequences, sometimes becoming severely introverted and reclusive (Gillberg and Winnergard, 1984; Gillberg and Steffenburg, 1989). Since social interaction occurs almost constantly in normal daily life, its avoidance can have a pervasive effect on an individual's human experience.

## 1.2 Treatment of Facial Palsy

### 1.2.1 Conservative & Medical Treatment

The treatment of facial palsy is determined by its cause and chronicity. Facial palsy of reversible cause is managed supportively with eye lubrication (artifical tears during the day and lubricating ointment at night) and by management of the underlying condition – for instance, corticosteroids and/or antiviral medications in Bell's palsy (Holland and

Weiner, 2004). Bell's palsy has an excellent prognosis with around three quarters of patients recovering completely (Holland and Weiner, 2004).

### 1.2.2 Surgery for Acute Facial Palsy

Facial palsy resulting from physical disruption to or agenesis of the facial nerve has no potential for spontaneous recovery. Surgery is required to restore symmetry and movement to the face.

#### a) Facial nerve repair

If possible, early repair of the facial nerve is the best treatment option. This can be achieved by direct repair or using an interposition nerve graft if there is segmental nerve loss. For success by nerve repair alone, the proximal part of the facial nerve must be available and the neuromuscular system still functional. As the interval between denervation and repair increases the functional result worsens (Aydin et al., 2004), until eventually after prolonged denervation muscle becomes irreversibly atrophic.

#### b) Cranial nerve substitution

When the proximal part of the facial nerve is unavailable, such as following tumour

resection, restoration of facial nerve continuity is not possible. In such instances reinnervation of facial musculature using a different cranial nerve can be considered. Donor nerves for cranial nerve substitution techniques have included the hypoglossal and spinal accessory nerves. A disadvantage of this approach is that the facial expression of spontaneous emotion ('mimic expression') is conveyed specifically via the facial nerve. Cranial nerve substitution may restore voluntary movement but it will be devoid of spontaneity.

In order to overcome this, wherever possible the facial nerve is used for reinnervation. If it is not possible to use the ipsilateral facial nerve then a branch of the contralateral nerve may be used via a nerve graft, as pioneered by Scaramella and Tobias (Scaramella and Tobias, 1973), Smith (Smith, 1971) and Anderl (Anderl, 1973).

### 1.2.3 Surgery for Chronic Facial Palsy

In chronic facial palsy none of these options are generally felt to be sufficient, due to inadequate functional response of chronically denervated muscle to reinnervation (Lundborg et al., 1994; Hua et al., 2003).

Various surgical approaches to chronic facial palsy have been devised and can be considered either static procedures, in which resting asymmetry is improved but no movement restored, or dynamic procedures which aim to restore symmetry both at rest

and on movement.

a) Static Procedures

Static procedures support and stabilise the soft tissue structures of the face but do not provide reanimation. Such procedures include gold weight upper eyelid loading, various lower lid procedures for ectropion (such as canthoplasty, lid tightening or lid suspension), oral commissure and lip suspension, nasal lateralization, cheiloplasty and various cosmetic procedures such as browlift, blepharoplasty and rhytidectomy (Terzis and Noah, 2003). They are an important option for debilitated or older patients for whom dynamic reconstruction may not be appropriate. Static procedures are often the procedures of choice for the eye in facial paralysis, and may be useful adjuncts to dynamic procedures to enhance facial symmetry.

b) Dynamic Procedures (Facial Reanimation)

Dynamic procedures refer to techniques that can restore movement to the face. In addition to restoring innervation to the paralysed face or hemiface, in chronic facial palsy there is the added challenge of replacing dysfunctional muscle with fresh muscle that can respond to nervous stimulation. These dual problems have been addressed by local muscle transfers, using non-facially innervated muscle such as masseter (Webster, 1956),

temporalis (Webster, 1956; Edgerton et al., 1975; Rubin, 1976; Morello and Converse, 1977; Puckett and Beg, 1978) and platysma (Edgerton et al., 1975). However the movement generated in this way was inelegant, asymmetrical and lacked spontaneity, and often a noticeable contour deficit was produced at the donor site.

An alternative approach was to import fresh muscle to the region. At first non-vascularised muscle grafts were used (Thompson, 1971), reliant on capillary in growth for nutrition and survival. As a result they suffered a significant ischaemic insult: central necrosis (Das et al., 1982) and atrophy (Frey et al., 1985) were common, associated with greatly weakened contraction (Guelinckx et al., 1984). Although some success was achieved using small muscles or muscle pieces (Gierup and Hakelius, 1978; Thompson, 1981), larger muscle units produced disappointing results (Miller, 1978; Nicolai, 1981).

The development of operating microscopes and microsurgical equipment in the 1960's made the rejoining of small neurovascular pedicles a technical possibility, permitting transfer of muscle and other tissues to distant parts of the body (Jacobson and Suarez, 1960). Pioneering work in canine rectus femoris demonstrated that revascularised free muscle flaps maintained near normal structure for at least 5 months, did not undergo early central necrosis and could generate useful work at the new site, in contrast to non-vascularised grafts (Tamai et al., 1970). This experimental work was rapidly followed up by functional muscle transfer in the clinical setting (Harii et al., 1976) and the efficacy of revascularisation in maintaining muscle structure subsequently confirmed by direct comparison between vascularised and non-vascularised muscle grafts (Frey, Gruber et al.

1985) (Guelinckx et al., 1984), establishing that microvascular anastomosis was essential for maintaining muscle structure and useful function after transplantation.

The process of donor muscle dissection, division of the neurovascular pedicle and re-establishment of the vascular and neural supply elsewhere in the body to perform useful contractile work has been termed functional muscle transfer (FMT). FMT combined with reinnervation by cross-facial nerve grafting (CFNG) has become the modern technique of choice for reanimation of the chronically paralysed face.

c) One- versus Two-Stage Functional Muscle Transfer and Cross-Facial Nerve Grafting

In the clinical setting most surgeons prefer to perform FMT with CFNG as two planned procedures in order to avoid potentially deleterious effects of FMT denervation atrophy occurring whilst axons regenerate across the nerve graft. This is based on the premise that axons, regenerating at a rate of approximately 1-2 mm/day (Kerns et al., 2003), will take up to six months to cross a 15-20cm CFNG. Since muscles denervated for one year[1] develop non-functional motor end-plates (Hua et al., 2003) and undergo irreversible atrophy (Lundborg et al., 1994), a six month denervation interval is considered a significant affront to FMT function, particularly in conjunction with other insults associated with distant transfer such as tenotomy, proprioceptive denervation and altered insetting tension (Frey et al., 1996). The denervation interval is therefore minimised by

[1] This may not be the case in humans, in whom restoration of function in atrophic muscle has been demonstrated 15 years post denervation. It is, at least, probably fair to say that there is permanently reduced functional recovery following prolonged denervation.

most surgeons by performing surgery over two stages and delaying FMT until axons have reached the distal end of the CFNG (Koller et al., 1993).

Despite a sound theoretical basis, evidence regarding both the critical duration of prolonged muscle denervation, and more directly, comparisons of one- and two-stage surgery have yielded conflicting results. Mass and force deficits in the rat gastrocnemius have been shown to be directly proportional to the denervation interval in one study (Aydin et al., 2004) whereas other results indicate that the rat tibialis anterior denervated for up to one year prior to reinnervation exhibited no greater force deficit than freshly reinnervated muscle, although a reduction in nerve regeneration capacity and a compensatory increase in motor unit size was noted to account for this (Fu and Gordon, 1995). In the rabbit, transection and repair of the rabbit peroneal nerve appeared to produce satisfactory outcomes after delays as long as ten months (Richter et al., 1979), and the true denervation interval that may be safely tolerated by human skeletal muscle is not known. Clinical comparisons between one- and two-stage facial reanimation by FMT with CFNG have found little difference between them, except for earlier onset of movement in the former (Harii et al., 1998; Kumar and Hassan, 2002) and improved static position in the latter (Kumar and Hassan, 2002). Since these were clinical series, muscle reinnervation or FMT force production could not be measured and the main outcome measures were semi-quantitative grading systems. Experimental comparisons of one- and two-stage surgery have concluded variably that two-stage surgery offers superior results (Rab et al., 2002) or, curiously from the same research group, that either technique produces similar outcomes (Rab et al., 2006).

One-stage surgery offers a number of potential advantages over two-stage surgery, including the desirability for both patient and surgeon to minimise the number of surgical procedures, shorter total recovery time (Harii et al., 1998; Kumar and Hassan, 2002), economic implications and potentially superior axon regeneration through the interposed nerve graft owing to neurotrophic and neurotropic effects of an attached end organ (Lundborg et al., 1982b; Lundborg et al., 1982a; Politis et al., 1982; Longo et al., 1983; Ochi, 1983; Hansson et al., 1986). In support of the latter, superior nerve regeneration[2] was demonstrated across sural nerve grafts coapted proximally to a facial nerve branch in monkeys with, compared to without, distal attachment to facial muscle (Mackinnon et al., 1988). In a more recent study, similar FMT outcomes in terms of tetanic force production, and superior outcomes in terms of motor nerve reinnervation, were obtained for one- compared with two-stage FMT with CFNG (Rab et al., 2006).

Despite this data, however, traditional surgical predilections for two-stage surgery have prevailed, with neither group recommending one-stage facial reanimation despite their own data. Instead, in their model of CFNG, Mackinnon and colleagues suggested that the distal nerve graft should be sutured to the distal facial nerve trunk on the paralysed side before performing FMT as part of a two-stage procedure (Mackinnon et al., 1988), although it is unclear whether coaptation to a distal stump attached to chronically denervated, dysfunctional atrophic facial muscle would exert the same neurotrophic and neurotropic effects. Rab and colleagues continued to favour two-stage surgery based on

[2] As indicated by mean axon areas of 3.08 $\mu m^2$ vs. 2.37$\mu m^2$, with fewer epineurial regenerating units.

reduced versatility and the need to create additional operative scars in one-stage surgery (Rab et al., 2006). This is derived from the practice of surgeons to perform one-stage facial reanimation without a nerve graft, instead using a FMT with a long motor pedicle, based on the belief that axons will not successfully cross a second coaptation if FMT and CFNG are performed at one stage (Anderl, 1973). As such, the choice of FMT is limited, its insetting range reduced and an additional scar needed to tunnel the neural pedicle. In keeping with clinical facial reanimation, the experimental model used by Rab et al. compared two-stage surgery using a nerve graft with one-stage surgery using a long neural pedicle and no nerve graft, and superior reinnervation outcomes were ascribed to the avoidance of a distal coaptation (Rab et al., 2006).

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production, and superior outcomes in terms of motor nerve reinnervation, were obtained for one- compared with two-stage FMT with CFNG (Rab et al., 2006).

## 1.3. Unpredictable Results of Facial Reanimation by Functional Muscle Transfer with Cross-Facial Nerve Grafting

The few long term, large series clinical publications on facial reanimation by functional muscle transfer (FMT) with cross-facial nerve grafting (CFNG) (O'Brien et al., 1990; Terzis and Noah, 1997; Chuang, 2002; Kumar and Hassan, 2002; Hamilton, 2003) and a number of smaller series (Gary-Bobo et al., 1980; Yla-Kotola et al., 2004) reveal a highly variable clinical outcome (McKee and Kuzon, 1989; Terzis and Noah, 1997). Comparison between series is difficult because of variable study populations and clinical outcome measures: for instance, Kumar used a 1-3 grading system, Terzis a 1-5 grading system, O'Brien a 1-4 grading system and Yla-Kotola a 1-6 grading system; and study populations were composed of 20%, 34%, 68% and 100% patients with complete facial palsy respectively. Notwithstanding these differences, Kumar (Kumar and Hassan, 2002) achieved a 92% fair or good outcome ('good' being the highest possible rating in this series), Terzis (Terzis and Noah, 1997) a 42% good or excellent outcome, O'Brien (O'Brien et al., 1990) a 51% fair to good outcome and Yla-Kotola (Yla-Kotola et al., 2004) a 59% mild or moderate residual dysfunction.

Collectively these figures suggest that approximately 10-60% of patients undergoing facial reanimation using FMT achieve inadequate or no movement postoperatively.

Moreover, a subgroup of patients initially classed as having good postoperative movement later develop facial distortion towards the operated side. Rates of 'muscle tightening' are quoted at 20-30% (Chuang et al., 1995; Chuang, 2002) and at 33% (Hamilton, 2003). O'Brien and Kumar describe a late muscle 'tethering' phenomenon with rates quoted at 59% (O'Brien et al., 1990) or 10-13% (Kumar and Hassan, 2002). Although attributed to scarring, 'tethering' was similar in time of onset and appearance to the dynamic tightening reported by Chuang and Hamilton (Chuang et al., 1995; Chuang, 2002; Hamilton, 2003).

Although numerous surgical variables (such as techniques for nerve coaptation, types and numbers of CFNG, CFNG routes and therefore graft lengths, and donor muscles) could contribute to clinical variability, highly variable outcome of FMT with CFNG persists within individual series. Some feel this unpredictability is sufficient to warrant planned revision surgery from the outset (Frey and Giovanoli, 2002). This is unsatisfactory for patients: the need for revision surgery is disappointing, places the patient under further operative risk, lengthens total recovery time and does not guarantee optimal outcome. Unpredictability of outcome also limits the potential usefulness of FMT for the treatment of conditions other than facial palsy.

Although many studies have compared denervated muscle recovery using different motor inputs, very few have used FMTs as recipient muscles, facial nerve branches as donor nerves and quantified muscle recovery by measuring force production. One such study, however showed that heterotopic FMT reinnervation and force production depend on the

magnitude of donor motor input (MacQuillan, 2005). In the New Zealand white rabbit rectus femoris FMT model, muscles reinnervated using the larger VRB facial nerve branch generated 150% absolute force of those reinnervated using MM., corresponding in the former with greater reinnervation, as determined by nerve morphometry at the neural pedicle.

FMTs reinnervated via an interposed nerve graft are believed on theoretical grounds to be highly sensitive to differences in reinnervating axon load, due to axon loss across the nerve graft and at proximal and distal coaptations, a relative paucity of reinnervating motor axons and concomitant formation of maximally-sized motor units (Urso-Baiarda et al., 2006). As a result, small random between-case differences in motor axon load reaching a FMT could have very large effects in terms of force production. Since reinnervation of a FMT with an interposed nerve graft involves random axon loss at both coaptations and along the graft itself, such a mechanism could contribute to a degree of inherent variability of force production by such a FMT. The corollary is that it may be possible to standardise outcome more accurately if it were possible to control FMT neurotisation more precisely.

## 1.4. Limiting FMT Neurotisation

### 1.4.1. Through Motor Input Selection

Despite evidence that FMT neurotisation and function may be dependent on the size of the reinnervating motor nerve (MacQuillan, 2005) there are several reasons why it is difficult to achieve consistent FMT neurotisation through donor nerve branch selection in the context of facial reanimation surgery. Firstly, motor nerve branches reinnervating a FMT must be selected on the basis of function rather than size – that is, if the FMT has been inset such that its shortening will elevate the corner of the mouth, a branch of the facial nerve whose stimulation elevates the contralateral corner of the mouth must be used as the donor nerve supply, irrespective of its size. Secondly, due to the extensive and random ramifications of facial nerve branches in the face (Kwak et al., 2004), there may be large size variations in the functionally correct facial nerve branch between individuals, so use of the same anatomical nerve branch in different cases will not necessarily guarantee the same donor motor axon supply. Thirdly, it is not accurately possible to know the axon content of a nerve branch intraoperatively since the necessary quantification is extremely time consuming (Urso-Baiarda and Grobbelaar, 2006). Finally, even if it were possible to have free choice of any donor nerve branch of known axonal content, neural escape at proximal and distal neurorrhaphy sites is to an extent random and uncontrollable, so axon supply may be dissimilar to axon delivery to the FMT.

In view of these objections to regulating FMT neurotisation by motor nerve branch selection, attention has turned to the possibility of manipulating axon transmission from donor nerve to FMT using the interposed nerve graft.

### 1.4.2. Through Low Capacity Interposed Nerve Grafts

It has long been recognised that reinnervation of muscle via a nerve graft results in greater force deficits than direct reinnervation by the motor nerve (Brooks, 1955; Hems and Glasby, 1992) provided that the latter may be achieved without placing the nerve repair under undue tension (Kim et al., 1990). It has also been recognised that some nerve grafts provide more favourable environments for transmission of axon regenerates than others - for instance motor nerve grafts permit superior regeneration compared with sensory grafts (Giovanoli, 1996). Thus nerve grafts may be conceptualised as having relatively high or low axon transmission capacity depending on their characteristics.

Characteristics known to affect axon transmission by a nerve graft include graft length. Evidence suggests that increasing nerve graft length correlates with worsening axon transmission capacity, first indicated by a retrospective clinical study demonstrating worsening functional outcome of nerve grafting with increasing graft length (Geldmacher and Albers, 1982). In that study it was unclear whether injury severity was a confounding factor since longer original nerve defects mandated longer grafts, and confirmation was sought in animal models in which it was found that 3, 5 and 7cm nerve grafts produced force deficits of 0.4%, 33.5% and 28.8% respectively (Rab et al., 2002), suggesting that short nerve grafts produced a better outcome than long or intermediate ones.

Axon diameter has also been considered a determinant of axon transmission. Following a literature review of causes of nerve graft failure, Brooks proposed certain principles that

should be adhered to for successful nerve graft repair including that the cross sectional area of the graft or grafts should equal that of the nerve to be repaired (Brooks, 1955). More recently, albeit without quantification, increasing physical size mismatch between a large proximal and small distal nerve stump was observed to result in increasingly large neuromas at the suture line, felt to result from loss of 'capture' of outgrowing regenerating nerve fibres by the distal stump (Mackinnon et al., 1989). Avoidance of axonal escape and improved muscle reinnervation could therefore be predicted by use of a larger calibre nerve graft.

The effect of nerve graft diameter on its ability to transmit axons may be indirect. When a nerve graft is harvested, severed axons and myelin degenerate and are phagocytosed, but Schwann cells survive (Aguayo et al., 1977) and aid the passage of regenerating axons both through the formation of physical conduits (Guth, 1956) or 'Schwann tubes' (Letourneau, 1975; Scherer and Easter, 1984) and the secretion of factors which promote growth of axon regenerates. Since Schwann cells appear to enhance peripheral nerve regeneration in a dose-dependent manner (Guenard et al., 1992) and a correlation between the number of Schwann cells, Schwann tubes and total nerve diameter are probable, the relationship between axon width and the capacity to transmit axons could be mediated by Schwann cell content.

A caveat to the realtionship between nerve graft diameter and axon transmission is that the largest diameter nerve grafts may suffer central ischaemic damage, lose cellular and structural composition and conduct axons poorly despite preserved physical proportions.

Some have recommended the use of vascularised nerve grafts in such cases based on improved axon regeneration through maintained Schwann cell populations and reduced fibroblast infiltration and endoneurial scarring (Terzis et al., 1995), although this technique has not been greatly adopted in the clinical setting. In the absence of ischaemia it is possible that graft diameter and Schwann cell content independently contribute to axon transmission capacity.

The presence of identifiable factors contributing to axon transmission capability by a nerve graft suggest that some types of nerve graft could limit axon transmission in a predictable manner. The surgeon could take advantage of this in facial reanimation by using low capacity nerve grafts to prevent fluctuations in FMT reinnervation, caused by random events and likely to contribute to the high variability of the procedure. For the purposes of this work, a 'low capacity' nerve graft is conceptually defined as one which is incapable of transmitting all the regenerating donor axons available to it (and therefore motor nerve supply reaching the distal end of the graft is dependent on the graft itself rather than the donor motor input). A 'high capacity' graft, conversely, is potentially able to transmit all regenerating donor motor axons to the distal end with relative high fidelity (and thus the motor nerve supply reaching the distal end of the graft is more dependent on the donor motor input than on the graft).

Identification of suitable high and low capacity nerve grafts to be compared in the rabbit model of functional muscle transfer used in the present study occurred by means of preliminary dissection and comparative morphometry. In seeking potentially useful nerve

grafts for comparison, desirable criteria were: two nerves of statistically dissimilar axon area and/or number; sensory nerves as opposed to motor nerves, in order to represent clinical practice more closely; nerve grafts which could be harvested via a thigh incision, in order to obviate additional scars and reduce animal morbidity; and a donor defect that was compatible with medium term survival, since final experiments were to take place after FMT recovery several months later.

Previous work using autologous nerve graft for muscle reinnervation in a rabbit model have made use of sural, posterior tibial (Matsuzaki et al., 2004), sciatic and saphenous nerve grafts (Koller et al., 1997), of which only the sural and saphenous were purely sensory and only the saphenous could be harvested via a thigh incision. In order to identify a second nerve graft of different neural content but similar length for comparison with the saphenous, exploratory dissection was performed in rabbit cadavers, through which it was discovered that a 3cm length of the much narrower medial branch of the saphenous nerve (SB) could also be obtained via the same thigh incision. Six biopsies of each of the saphenous and medial saphenous nerves were compared histomorphometrically and confirmed their suitability for use in this study. These results are detailed in Chapter 4 (Section 5)

## 1.5. Quantification of Axon Regrowth

### 1.5.1. Introduction

Histopathological examination of peripheral nerves is often quantified by morphometric analysis, and the current experimental work has a very high requirement for such morphometry. Previously published methodologies for this task are either fully manual or involve varying degrees of automation.

There is inconsistency in the literature regarding the terminology applied to different methods of morphometry (Mathews, 1968; Sharma and Thomas, 1974; Ellis et al., 1980; Friede and Beuche, 1985; Vita et al., 1992; Mezin et al., 1994; Koller et al., 1997; Romero et al., 2000). In this discussion, the terms are used as follows: 'manual' morphometry to refer to the use of a ruler, grid or scale to measure parameters from an image; 'semi-automated' morphometry to refer to the use of a digitising tablet, mouse or digital pen to trace around either printer or electronic nerve images whilst a computer measures or calculates values from this outline; 'fully-automated' morphometry to refer to morphometric assessment requiring no user input during the process; and 'interactive automated' morphometry to refer to automated morphometry which utilises human editing of the analysed image.

### 1.5.2. Existing Morphometric Techniques

Manual measurement of morphometric characteristics of nerve biopsies permit complete user control over what is and is not measured, theoretically offering optimal quality control over data analysis and avoiding both false inclusion and exclusion of image features. Despite this, manual morphometry has been largely abandoned in favour of

newer techniques. As well as being highly labour intensive, significant error in area assessment by manual nerve morphometry arises from the need to calculate axon area from the area of a circle of equivalent diameter. Auer points out that in distorted (flattened or crenated) nerve fibres the area derived from a minimum diameter calculation is a significant underestimate (Auer 1994). This is also the case for any obliquely sectioned fibres (with oval outlines), where a choice must be made between measuring a large diameter, overestimating area, or a small diameter, underestimating fibre density (due to the smaller number of fibres per unit area in oblique sections).

Semi-automated morphometry permits more precise area assessment based on the exact axon or fibre perimeter rather than a circular estimate of it. However, both manual and semi-automated nerve morphometry are considered extremely boring and time consuming, '...involving considerable tedium and time-related cost' (Auer, 1994) and that the requirement for prolonged manual attention may attract error. This is, however, difficult to quantify in the absence of an alternative gold standard technique.

Operator fatigue, economic and time considerations may mandate sampling of only a proportion of fibres when either method is used, and consequently there have been several attempts to devise representative sampling schemes (Mathieu et al., 1981; Müller et al., 1981; Mayhew and Sharma, 1984a, 1984b). Such attempts have been hampered by the bimodal size distribution and extremely heterogeneous local densities of nerve fibre populations (Torch et al., 1989b). In order to sample accurately, not only must each

subpopulation (large and small myelinated fibres) be accurately represented but their relative proportions must also be known.

Whilst isolated studies report good mean peripheral nerve fibre diameter estimation from small sample sizes (e.g. 185/2900 fibres) (Mayhew and Sharma, 1984b), others indicate that at least 50% sampling is necessary to achieve reasonable area and perimeter estimations (Torch et al., 1989b).

The main advantages of fully automated morphometry are, through avoidance of human input, loss of subjective decision-making and an increase in processing speed, which may in turn obviate the need for sampling. For instance, average processing speeds of approximately 1.5-10 seconds per analysed nerve fibre are quoted for fully automated methods (Ellis et al., 1980; Selva et al., 1981; Romero et al., 2000) compared with 20-40 seconds per analysed nerve fibre for semi-automated methods (Mezin et al., 1994; Romero et al., 2000). However, increased processing speed appears to take place at the expense of accuracy with fully automated nerve morphometry. Algorithms used to discriminate nerve fibres from background or artefactual features may not work from time to time, and without human quality-control uncorrected errors in image interpretation are converted to spurious data, estimated at around 9% (Usson et al., 1991).

A common error in automated nerve morphometry is the interpretation of close or overlapping 'particles' as single particles. Another source of error in automated nerve morphometry is the false positive, caused by inappropriate recognition of artefact such as

excessive stain, or of background features such as connective tissue, blood vessels, blood cells or degenerate axons. A third type of error is missed detection of true axons, arising because algorithms typically reject rather than correct maldetected axons such as those with a break along their boundary (Ellis et al., 1980). Several authors have remarked on the underestimation of numbers of small or regenerating axons by automated morphometry (Ellis et al., 1980; Usson et al., 1991; Vita et al., 1992) since their thin, poorly-staining myelin provide insufficient contrast with the interstitial space for automatic detection.

Few studies have attempted to quantify the error encountered with the use of fully automated morphometry. One study, however, compared their fully-automated morphometric technique with a standard semi-automated approach[4] for a single cat sciatic nerve biopsy (Romero et al., 2000). In that study, 3434 nerve fibres were detected manually compared with 3256 detected automatically (94.8% of semi-automated count), from which 33 (1.0%) were considered false detections and 211 (6.1%) considered missed detections. It is not possible to know whether other automated methods, or indeed increased sample sizes using the previous method, would achieve that degree of accuracy.

Other evidence suggests that this may not be the case. Comparison of fibre numbers analysed using fully-automated and interactive-automated methods has revealed differences of 14.4% (Selva et al., 1981) and 16% (Savy et al., 1987), whilst a comparison of myelinated fibre number analysed using interactive automated and semi-

[4] This paper used different morphometry terminology, describing semi-automated morphometry using a digital image and mouse as 'manual'.

automated morphometry[5] displayed a non-significant 0.8% difference and 0.6% inter-observer variability between two users performing five repeats (Mezin et al., 1994). Since the correlation between interactive-automated and semi-automated methods is high (Mezin et al., 1994), moderate discrepancies in the studies by Selva and by Savy comparing fully-automated and interactive-automated techniques are more likely to reflect error inherent to full automation.

Improved accuracy of interactive automated morphometry compared with the fully-automated approach does not appear to be at the detriment of sampling rate, with reported rates of 3.5-7 seconds per analysed nerve fibre (Torch et al., 1989a; Mezin et al., 1994), similar to those published for fully-automated nerve morphometry. This may be due to the need for human intervention for only a small proportion of axons, allowing average analysis rates to approximate those of fully automated morphometry.

However, other impediments to the use of fully automated morphometry apply equally to interactive-automated morphometry. Both require dedicated laboratory space, specialised equipment and therefore significant set-up costs (Zimmerman et al., 1980). Automated or interactive automated processes can also be difficult to implement because their methodological description in the literature is often in conceptual or mathematical rather than practical terms (Ellis et al., 1980), (Romero et al., 2000). Since these methods use custom-made software that is not widely available, reproduction of the described

[5] Similarly, this paper described interactive automated morphometry as 'semi-automated' and semi-automated morphometry using a digitising tablet as 'manual'.

methodology would require the *de novo* production of software based on the principles described.

### 1.5.3. The Need for a Modified Morphometric Technique

Existing methods of nerve histomorphometry therefore required modification to obviate the need for specialised equipment, sufficiently automated to prevent the need for sampling whilst remaining accurate enough to produce reliable data. In view of this, the current method of nerve morphometry was developed (Urso-Baiarda and Grobbelaar, 2006) aiming to fulfil the following principles:

A fully-automated component for rapid evaluation of most axons;
A semi-automated component for slower evaluation of remaining axons and error correction;
A software-based approach avoiding the need for specialised hardware; and
The use of non-proprietary publicly available software for more widespread applicability of the method.

In order to assess the validity of this method, it was compared with a semi-automated method typical of those in most popular use for nerve morphometry (Urso-Baiarda and Grobbelaar, 2006).

## 1.6. Hypotheses

Since previous studies experimental work has shown that force production achieved by a FMT is dependent on the magnitude of donor motor input, and on theoretical grounds FMTs (or any muscle reinnervated with greatly reduced axon load) appear to exhibit increased sensitivity to variations in motor input received, random variations in reinnervation dose received by a FMT could contribute to the highly variable clinical outcome observed. The corollary is that such variation could be reduced by achieving more consistent FMT neurotisation between cases.

It was hypothesised that a high capacity interposed nerve graft would permit relatively faithful regeneration of axons from motor nerve to target organ, whereas a low capacity nerve graft would impair neurotisation both of the graft itself and of any coapted end organ. Essentially, by limiting axon regeneration across it, a low capacity nerve graft could improve inconsistency in FMT outcome caused by variations in donor nerve input.

## 1.7. Study Aims

The overall aim of this research project was to assess the potential effect of low capacity nerve grafts to standardise axon delivery to FMTs in order to regulate their force production despite variable motor input. With this aim, a New Zealand white rabbit model of FMT with CFNG was compared in terms of reinnervation and force production

using either a large or small donor nerve combined with either a high or low capacity interposed nerve graft. This was repeated for both one- and two-stage surgery groups, since it was felt that the results of one could not reliably be extrapolated to the other, and in the clinical setting neither is unequivocally accepted as gold standard. Since each experimental one-stage group had a corresponding two-stage group, and in light of the controversy regarding the preferability of one- or two-stage surgery in the clinical setting, a secondary study aim was to compare one- and two-stage approaches to FMT with CFNG.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Muscle Transfer Model

An animal model was used to investigate the effects of reinnervation using high or low donor motor axonal load combined with Schwann tube-rich or –poor interposed nerve grafts on reinnervation and force production by FMTs. The New Zealand white rabbit rectus femoris FMT model was selected for the experimental work because of its favourable anatomy (including a single neurovascular pedicle and a distinct tendinous origin and insertion), a non-crippling donor site defect compatible with a long experimental recovery time, and previous experience at our research unit (Grobbelaar, 2003; MacQuillan, 2005) and extensively in the literature (Terzis et al., 1978; Frey et al., 1983a; Guelinckx et al., 1984; Frey et al., 1985; Frey, 1998; Giovanoli et al., 2003). All operations were carried out in accordance with Home Office guidelines.

## 2.2. Study Groups

It was necessary to adopt a multivariate experimental design with all experiments running simultaneously owing to the long duration of individual experiments necessary for FMT recovery. Three variables were investigated: donor motor input; interpositional nerve graft diameter; and one- versus two-stage surgery. For simplification all were treated as binary rather than continuous variables (large or small donor nerve and wide or narrow nerve graft). Thus there were a total of 8 study groups between which 40 animals were divided equally (Table 2.1). In each case, the left rectus femoris was transferred to

**Table 2.1.** Experimental groups.

| | Study Group | Donor facial nerve branch | Nerve graft |
|---|---|---|---|
| One-stage | 1 | Marginal mandibular br. | Medial branch of saphenous n. |
| | 2 | Marginal mandibular br. | Saphenous n. |
| | 3 | Ventral ramus of buccal br. | Medial branch of saphenous n. |
| | 4 | Ventral ramus of buccal br. | Saphenous n. |
| Two-stage | 5 | Marginal mandibular br.. | Medial branch of saphenous n. |
| | 6 | Marginal mandibular br. | Saphenous n. |
| | 7 | Ventral ramus of buccal br. | Medial branch of saphenous n. |
| | 8 | Ventral ramus of buccal br. | Saphenous n. |

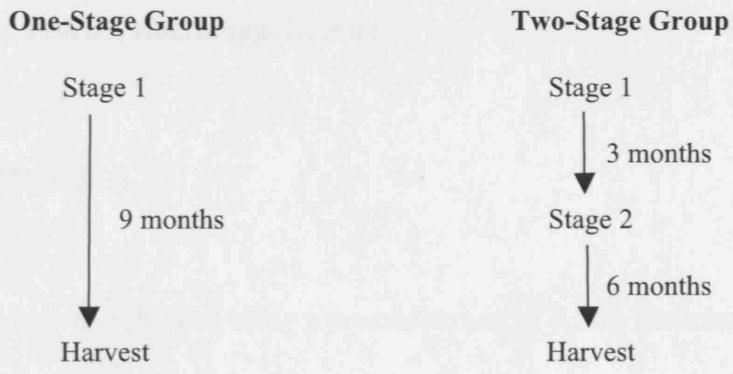



**Figure 2.1.** Timing of operative procedures. Muscle harvesting took place 9 months after the first stage for animals undergoing either one-stage or two-stage surgery.

the right hemiface and the non-transferred contralateral rectus femoris muscle of each rabbit was used as its own internal control.

## 2.3. Operative Technique

The four groups allocated to one-stage surgery underwent left rectus femoris transfer to the right cheek, restoration of vascular continuity and reinnervation from the right facial nerve branch via a 30mm harvested nerve graft as a single procedure. Those allocated to two-stage surgery first underwent 30mm nerve graft harvesting and coaptation to the donor motor branch of the right facial nerve. The free distal end of the nerve graft was marked with 10/0 nylon (S&T, Neuhausen, Switzerland) and left free in the right cheek. During the second stage, performed three months later, the left rectus femoris muscle was transferred to the right cheek. All neurovascular work including vessel dissection, vascular anastomosis and neurorrhaphy was performed with the aid of an operating microscope (Wild, Heerbrugg, Germany).

### 2.3.1. Preparation

Anaesthesia was achieved using a premedication of 0.7ml intramuscular Hypnorm (Janssen, Oxford; fentanyl citrate 0.15mg/ml; fluanisone 10mg/ml) followed by 0.4ml intravenous diazepam (Hameln Pharmaceuticals Ltd.) via a cannulated ear vein (Figure 2.2). Intravenous Hypnorm was administered as required for maintenance anaesthesia. During surgery the animal was given 2 litres/minute oxygen by facemask and was kept warm with an electrical warming mattress placed beneath the operating board. All

**Figure 2.2** Induction of anaesthesia in animals. (a) The animal is wrapped in a blanket after sedation with intramuscular Hypnorm and the dorsal ear vein is (b) shaved and (c) cannulated. Anaesthesia is then induced using intravenous diazepam.

**Figure 2.3.** Animal preparation and positioning for surgery. (a) Shaved left leg. (b) Shaved right side of face. (c) The animal is positioned supine with the head turned to the left and the left leg abducted, extended and externally rotated. (d) and (e) The face and leg are sprayed with 0.5% chlorhexidine gluconate and shielded with clean surgical drapes.

animals received 10–15ml intravenous normal saline during their procedures and 30ml subcutaneous normal saline at the end of the procedure.

The operative field was shaved using electrical clippers immediately pre-operatively and sprayed with 0.5% chlorhexidine gluconate (Hydrex, Adams Healthcare Ltd.). The animal was laid supine with the left hip extended, abducted and externally rotated and the face turned to the left, before draping to expose the left thigh and right side of face (Figure 2.3).

### 2.3.2. One-Stage Surgery

#### a) Nerve harvest

The main saphenous nerve or its medial branch, as required, was approached by an incision from the medial aspect of the patella to the midpoint of the thigh as far proximal as the abdominal wall. Subcutaneous fascia was cleared and medial and lateral skin flaps raised by blunt dissection and gently retracted using tissue forceps. This exposed the distal part of the saphenous vein as it became superficial at the medial aspect of the knee. The saphenous vein ran superomedial to the saphenous nerve and was used as a useful landmark. Using microscissors, connective tissue overlying the saphenous vein at the knee was divided, the saphenous nerve was located and followed proximally to its femoral nerve origin. At this point the medial saphenous branch leaves the main branch, crossing superficial to the saphenous vein and continuing parallel and medial to it.

Depending on the study group, a 30mm length (as measured in situ) of the proximal end of either saphenous nerve or medial saphenous nerve was harvested, its proximal end marked with a loop of 10/0 nylon (S&T, Neuhausen, Switzerland), and bathed in normal saline until required (Figure 2.4). Care was taken to ensure that nerve graft length was a constant 30mm for all animals since previous work has shown graft length to affect functional outcome (Rab et al., 2002).

b) Facial nerve grafting

A curved incision was made from the base of the ear to the mid-point of the inferior border of the mandible. The correct plane of dissection was identified superficial to masseter and the skin flap elevated to the inferior margin of the orbit, exposing the facial nerve including the buccal and marginal mandibular branches. The ventral ramus of the buccal (VRB) branch of the facial nerve was identifiable as a broad ribbon-like nerve whereas the marginal mandibular (MM) branch was a slender unbranching nerve positioned more inferiorly and readily identifiable by one or two small accompanying blood vessels. The donor nerve branch was dissected from surrounding tissue, divided and coapted to the nerve graft using 10/0 Ethilon (Figure 2.5).

Clinically, facial reanimation surgery by FMT requires an interposed cross-facial nerve graft (to bridge the gap between ipsilateral FMT and contralateral functioning facial nerve branch). In this animal model the FMT was reinnervated by an ipsilateral facial nerve branch since: a) the facial anatomy of the rabbit would mandate an excessively

**Figure 2.4.** Nerve graft harvest. (a) An incision is made along the anteromedial thigh and (b) medial and lateral skin flaps raised, (c) exposing the saphenous vein (SV). (d) The saphenous nerve (SN), located deep and lateral to the vein, is followed proximally to the origin of the medial saphenous branch (MSN) in the proximal thigh. A 3cm length of either nerve is then harvested.



**Figure 2.5**. a) The facial nerve is exposed and b) the facial nerve branch (MM or VRB) dissected and coapted to the nerve graft; (NG) c) This is tacked loosely to underlying masseteric fascia and the approximate position of the distal end marked with a nylon suture.

long nerve graft to use a contralateral branch, and b) the side of the reinnervating facial nerve branch used was not considered a factor affecting FMT outcome. In support of this, in a comparison of nerve regeneration across either ipsilateral or crossover nerve grafts in rabbits yielded similar medium-term results, although early results favoured ipsilateral grafting over crossover grafting, presumed to be due to greater mechanical stress in the latter (Frey et al., 1996).

Whilst in the clinical setting there is just sufficient nerve graft length to prevent tension across the neurorrhaphies, in this model there was considerable nerve graft redundancy in the cheek, which was loosely tacked into a wide arc. A previous histomorphometric comparison of nerve fibre regeneration across rat sciatic nerve isografts arranged in various geometries found no significant difference in nerve fibre number or area 6 weeks after regeneration across nerve grafts arranged with or without redundancy and in a sinusoidal or omega configuration, suggesting that nerve graft morphology does not influence axon regeneration (Kawamura et al., 2005)

Several studies have investigated the effect of nerve graft orientation on nerve fibre regeneration, finding that axons may be lost into nerve graft branches if it is inset in normal orientation but that this is prevented by insetting in reversed orientation (Ansselin and Davey, 1988). Since reversing a nerve graft has no adverse effect on axon regeneration (Stromberg et al., 1979; Ansselin and Davey, 1988; Sotereanos et al., 1992; Nakatsuka et al., 2002), all nerve grafts were inset in reversed orientation in this study.

c) Rectus Femoris harvesting

The rectus femoris muscle was harvested as described previously (MacQuillan and Grobbelaar, 2003) (Figure 2.6). The subcutaneous fascia overlying the quadriceps muscle was divided and the inguinal fat pad dissected free through the leg incision used for nerve graft harvest. A leash of significant blood vessels running between the medial end of the inguinal fat pad and overlying subdermal fascia could be retracted medially to assist the approach.

Adequate skin flaps for access to the inguinal region were dissected and the abdominal wall retracted superiorly to reveal the inguinal ligament. This was dissected from the thigh muscles using scissors and retracted superiorly with tissue forceps, permitting full-length exposure of sartorius (medially) fused with tensor fascia lata (laterally). The avascular plane between these muscles, visible as a longitudinal white line, was incised three quarters along its length from distal to proximal to expose the underlying rectus femoris. This was dissected from its muscular pocket by blunt dissection and the distal insertion into the patellar tendon was isolated.

More proximally, dissection between sartorius and tensor fascia lata continued using an operating microscope to prevent damage to the underlying venous pedicle, faintly visible as a blue line running parallel to the inguinal ligament. Microscissors were used to remove a thin layer of muscle immediately superficial to the venous pedicle, and the plane between sartorius and tensor fascia lata developed by blunt dissection using

**Figure 2.6.** a) An incision is made between sartorius and tensor fascia lata and b) rectus femoris is peeled off of these muscles. c) Careful blunt dissection around a faint visible blue line at the proximal end of the muscle, representing the venous pedicle, is carried out until d) this vessel has been cleared. The incision between sartorius and tensor fascia lata can then be

microforceps. In this way it was possible to identify the venous pedicle, the deeper arterial pedicle and the perpendicular motor nerve entering rectus femoris. The medial boundary of dissection of the pedicle was the femoral nerve (adjacent to the femoral vein and artery), and the lateral border the medial surface of vastus lateralis (fused with the overlying tensor fascia lata). Arterial and venous branches entering vastus medialis were identified and tied using 7/0 silk close to that muscle, and the pedicle to rectus femoris gradually isolated from neighbouring muscles and femoral nerve branches. Dissection of the pedicle was facilitated by gentle traction to the rectus femoris (applied laterally) and vastus medialis (applied medially), separating them and maximally exposing the neurovascular pedicle between them.

The lateral circumflex femoral artery was tied off and divided on the lateral border of the rectus femoris muscle prior to its bifurcation into ascending and transverse branches. The vein was also tied and divided at the lateral edge of rectus femoris, taking care to include within the pedicle a large anterior venous branch running superficially along the muscle (which was sometimes the dominant venous drainage for the flap) (MacQuillan and Grobbelaar, 2003). Secondary pedicles entering the lateral surface of the rectus femoris (either from muscular branches to the vastus lateralis or directly from the ascending branch of the lateral circumflex vessels) were often encountered but were small and easily divided with haemostasis using diathermy alone.

Once the neurovascular pedicle had been isolated, dissection continued proximally to release the remaining attachments of rectus femoris to surrounding muscles, so that the

muscle was attached only by its origin, insertion and neurovascular pedicle. The pedicle was bathed in procaine, the muscle wrapped in saline-soaked gauze and the skin flaps over it closed temporarily using haemostats.

d) Heterotopic muscle transfer

Attention then turned to preparation of the facial vessels. The facial artery and vein cross the lower border of the mandible at the angle of the jaw, the vein more superficial than the artery. These were dissected from the angle of the jaw working distally, dividing several small branches along the way, until 4-5 cm length had been dissected. The vessels were occluded with microclamps proximally, ligated distally and divided close to their point of ligation to preserve as much vessel length as possible. They were flushed with heparinised saline, bathed in procaine and covered with a saline-soaked gauze swab while the rectus flap was completely elevated.

The rectus femoris muscle belly was marked with a length of 3/0 braided silk (Ethicon, PO Box 408, Bankhead Avenue, Edinburgh, UK) to guide insetting at the recipient site at the correct resting tension. The insertion of the rectus femoris into the patella was then divided and a haemostat attached to the cut tendon end. This was used to apply gentle downward traction to the rectus whilst the origin was divided by following the muscle under the inguinal ligament with the blades of curved scissors. During this process the pedicle was wrapped in a sheet of visibility background material and retracted medially and inferiorly. The safety of the neurovascular pedicle could be guaranteed by keeping

the scissors beneath this background material at all times. Finally, the lateral circumflex vessels and the nerve to the rectus femoris were divided as close as possible to their origins to provide the maximal pedicle length.

The freed muscle was wrapped in saline-soaked gauze leaving only the pedicle exposed, and moved to the face. Using microneurovascular techniques the venous anastomosis was completed first, followed by the arterial anastomosis and finally coaptation of the free end of the nerve graft to the motor nerve to rectus femoris (Figure 2.7).

e) Rectus Femoris insetting

The flap was inset using 2/0 braided silk (Ethicon, PO Box 408, Bankhead Avenue, Edinburgh, UK) into the periosteum of the angle of the mandible and the auricular cartilage, at a resting tension which restored the length of silk suture within the muscle belly to its pre-transfer length (after which the suture was removed). The flap was anchored to fascia midway along its superior and inferior edges using 3/0 silk. Both facial and thigh wounds were closed using 3/0 braided silk (Ethicon, PO Box 408, Bankhead Avenue, Edinburgh, UK) horizontal mattress sutures.

**Figure 2.7.** (a) The facial vessels are dissected from the lower border of the mandible and (b) anastomosed to the vessels of the transferred rectus femoris muscle. The free end of the nerve graft is coapted to the muscle nerve and (c) the muscle is inset into auricular cartilage and mandibular periosteum. (d & e) Finally, the facial and leg wounds are closed and the animal recovered. (FV=facial vein; FA=facial artery; AA=arterial anastomosis; VA=venous anastomosis; N=neurorrhaphy.)

f) Postoperative

All animals received 0.3ml of subcutaneous Carprofen (Pfizer Inc., Zurich, Switzerland) and 30ml subcutaneous normal saline, were wrapped in a blanket and observed until they had recovered from anaesthesia sufficiently to be returned to their cage.

### *2.3.3. Two-Stage Surgery*

Preoperative preparation and postoperative care were as described above for animals undergoing two-stage surgery (Sections 2.4.1 and 2.4.2f). The first stage consisted of nerve graft harvesting and coaptation to the facial nerve branch (Sections 2.4.2a-b). The second stage was performed 3 months later and consisted of transfer of the rectus femoris to the face, microsurgical revascularisation and end-to-end neurorrhaphy of the distal end of the nerve graft to the FMTs neural pedicle (Sections 2.4.2c-e). Localisation and dissection of the nerve graft from surrounding scar tissue was facilitated considerably by the marker stitch placed near its distal end during the first stage.

## 2.4. Timing of Operative Procedures

Animals underwent either one or two-stage surgery. The interval between the first operation (i.e. the proximal nerve coaptation) and muscle harvest was 9 months, regardless of whether surgery was performed over one or two stages in total (Figure 2.1).

The principle aim of the one- versus two-stage study groups was to determine the effect of delayed reinnervation versus potentially enhanced axonal regeneration on FMTs (discussed in Chapter 6).

Three months was chosen as a suitable interval between stages for animals undergoing two-stage surgery since previous work has demonstrated an axon regeneration rate of 1.5mm/day across a fresh nerve graft in the rat (Kerns et al., 2003), and 3 months has previously been shown to be adequate for regenerating axons to cross a 28cm saphenous nerve graft without distal connection in the sheep (Frey et al., 1996). On this basis a three month interval between first and second stages was considered more than sufficient for regenerating axons to cross the 30mm nerve graft, even allowing for differences in the animal model and motor nerve used.

The presence of regenerated axons at the distal end of the nerve graft at three months was confirmed histologically in the two-stage group from nerve graft biopsies taken at the time of second stage surgery (See Chapter 4 Section 4.6).

## 2.5. Muscle Harvest

At the time of muscle harvest, animals were anaesthetised as before (Section 2.4.1) and the FMTs exposed through the previous facial operative incision. Particular care was taken to identify and protect the neurovascular pedicle, usually encased in scar tissue.

Once identified, the entire flap was elevated on its neurovascular pedicle and physiological assessment was carried out (Section 2.6). The contralateral (right thigh) rectus femoris was then dissected as described previously (Section 2.4.2c), except that the neurovascular pedicle was left intact and only the distal insertion of the muscle into the patellar tendon was divided. Physiological assessment was repeated for this control muscle.

## 2.6. Muscle Physiology

Physiological assessment was performed 9 months after the initial procedure to assess FMT force production.

### 2.6.1. Animal Preparation

Animals were anaesthetised as before (Section 2.4.1) and secured to a custom-made operating board (MacQuillan, 2005), itself firmly attached to the operating desk.

### 2.6.2. Electrical Stimulation of FMTs

Having exposed the muscle transplant and identified its neurovascular pedicle, the end of the muscle transplant previously inset to auricular cartilage (the distal end of muscle as orientated in its native position in the thigh) was dissected free and connected to a Grass FT10 isometric force transducer (Grass Technologies, West Warwick, USA) by means of

a 2/0 braided silk (Ethicon Inc., Somerville, New Jersey, USA) modified Kessler suture through the patellar tendon. The other end of the muscle was connected to a fixed retort stand with a 2/0 braided silk modified Kessler suture. The force transducer was connected to an ADInstruments semi-rectified bridge amplifier and an ADInstruments Power Lab 2/20 (ADInstruments, Oxfordshire, UK), in turn connected via a USB cable to a laptop PC running Chart for Windows v4.2 (ADInstruments, Oxfordshire, UK) software for recording force output measurements. During stimulation the muscle was kept wet using normal saline solution warmed to 37°C.

Platinum stimulating electrodes (Grass Instrument Division, Astro-Med Inc., 600 East Greenwich Av., West Warwick, RI, USA) held 5mm apart in a custom-made block and connected to a Grass S88 nerve stimulator (Grass Instrument Division, Astro-Med Inc., 600 East Greenwich Av., West Warwick, RI, USA) (Figure 2.8) were applied to the surface of the nerve graft mid-way along its length. The optimal resting tension for each muscle was assessed by measuring twitch contracture force over the range 100-170g tension on the muscle, with the tension producing the greatest twitch contracture used to carry out subsequent stimulation.

Force frequency curves were plotted by measuring muscle force generated against stimulation frequency, increasing from 10-100 Hz at 10 Hz intervals, using a constant pulse duration (0.2ms), stimulation period (1s), interstimulatory gap (10s) and

**Figure 2.8.** Electrical muscle stimulation: a) apparatus, NS = nerve stimulator, PL = Powerlab recording instrumentation; b) stimulating electrode (E) applied to motor nerve (N); c) stimulation of control rectus femoris (RF) and d) of FMT.

| Stimulation frequency (Hz) | Pulse duration (msecs) | Voltage (V) | Period of stimulation (secs) | Interstimulatory gap (secs) |
|---|---|---|---|---|
| 100 | 0.2 | 10 | 1 | 10 |
| 100 | 0.2 | 10 | 1 | 10 |
| 100 | 0.2 | 10 | 1 | 10 |
| 10 | 0.2 | 10 | 1 | 10 |
| 20 | 0.2 | 10 | 1 | 10 |
| 30 | 0.2 | 10 | 1 | 10 |
| 40 | 0.2 | 10 | 1 | 10 |
| 50 | 0.2 | 10 | 1 | 10 |
| 60 | 0.2 | 10 | 1 | 10 |
| 70 | 0.2 | 10 | 1 | 10 |
| 80 | 0.2 | 10 | 1 | 10 |
| 90 | 0.2 | 10 | 1 | 10 |
| 100[†] | 0.2 | 10 | 1 | 10 |

**Table 2.2.** Stimulation protocol carried out in sequence from top down for each muscle (both transplants and controls).

[†] Force fatigue rate measurement.

supramaximal stimulation voltage of 10V (Frey et al., 1996; Giovanoli et al., 2003; MacQuillan, 2005) (Table 2.2). A supramaximal 100Hz stimulation frequency[6] was finally used to measure tetanic force generation and fatigue rate.

A similar procedure was followed for physiological assessment of control (in-situ) rectus femoris in the right thigh, in that the muscle was elevated on its pedicle and direct nerve stimulation also performed. However, instead of detaching the pelvic origin, the pelvis was strapped rigidly to the operating desk. The patellar insertion was divided and connected to a force transducer as previously.Having performed physiological assessment of both transplanted and control rectus femoris muscles, the animal was euthanased using intravenous Lethobarb (pentobarbital sodium) (Fort Dodge Animal Health, Southampton, UK).

## 2.7. Physical Characteristics of Transplanted Muscle

After muscle stimulation the neurovascular pedicle was divided close to muscle and the muscle removed. Physical characteristics measured were weight, length and volume by liquid displacement. The facial nerve branch, nerve graft and muscle nerve were removed in continuity, divided at the two coaptation sites and fixed in PIPES buffered 2.5% glutaraldehyde solution (Box 2.1) for subsequent processing.

[6] muscle tetany typically occurring at 40–50Hz.

| Reagents | |
|---|---|
| PIPES (piperazine-N-N'-bis(2-ethane sulphonic acid)) | 3.024 g |
| 1M NaOH | 18ml |
| Distilled water | to 100ml |
| ***Method*** | |
| Add solid to approximately half the required volume of water, followed by the NaOH and stir to dissolve. Make up to 100ml in a measuring cylinder. Adjust pH to 7.6 (keep in refrigerator). To make fixative: add 2.5ml 10% glutaraldehyde and 7.5ml PIPES buffer, mix well (store in refrigerator). | |

**Box 2.1.** Preparation of PIPES buffered 2.5% gluteraldehyde solution

| ***PIPES buffered 2% $OsO_4$ + 3% $K_3Fe(CN)_6.3H_2O$*** | |
|---|---|
| $OsO_4$ | 2g |
| $K_3Fe(CN)_6.3H_2O$ | 3g |
| PIPES buffer | to 100ml |
| ***PIPES buffered 6% $NaIO_3$ + 4% sucrose*** | |
| $NaIO_3$ | 6g |
| Sucrose | 4g |
| PIPES buffer | to 100ml |

**Box 2.2.** Preparation of osmicating solutions

## 2.8. Nerve Histology Preparation

Fixation, staining and sectioning techniques employed were similar to those used in previous FMT studies and others looking at nerve grafting and nerve morphology (Koller et al., 1997; Nehrer-Tairych et al., 2000; Rab et al., 2002; Giovanoli et al., 2003). Immediately after the physiological assessment of the transplanted flap, the nerve graft and rectus femoris motor nerve were removed and divided at the distal neurorrhaphy site.

Immediately after harvesting, nerve biopsies were stretched out on a piece of labelled white card and fixed in PIPES buffered 2.5% glutaraldehyde solution (Baur and Stacey, 1977) overnight. Biopsies were then washed in PIPES buffered 2% sucrose solution for 30 minutes and placed in a 1:1 mixture of PIPES buffered 2% $OsO_4$ + 3%$K_3Fe(CN)_6.3H_20$ and PIPES buffered 6% $NaIO_3$ + 4% sucrose to osmicate the biopsies (Box 2.2). They were again left in this solution overnight. Biopsies were next sequentially dehydrated in alcohol (Box 2.3) and impregnated with epoxy resin (Box 2.4) following a previously published protocol (Dalley and Seliger, 1980; Langford and Coggeshall, 1980).

After dehydration and impregnation with resin overnight, nerve biopsies were embedded in fresh epoxy resin and placed individually in rubber moulds with an identifying label. These were left overnight in an oven at 66°C and a vacuum pressure of 15psi. Finally, the resin blocks containing the samples were removed from the moulds and any excess trimmed off, ready for sectioning.

| Solution | Wash time | Repeat washes |
|---|---|---|
| 15% ethanol | 5 mins | 2 |
| 30% ethanol | 10 mins | 2 |
| 50% ethanol | 15 mins | 2 |
| 70% ethanol | 30 mins | 2 |
| Dehydrated ethanol | 20 mins | 3 |
| Dehydrated ethanol | 60 mins | 3 |
| 1, 2 epoxy propane | 15 mins | 3 |
| Epoxy resin + 1,2 epoxy propane 1:1 | 60 mins | 1 |
| Epoxy resin + 1,2 epoxy propane 3:1 | Overnight | 1 |
| Epoxy resin | Overnight | 1 |
| Embed in epoxy resin | 24 hours at 66°C | - |

**Box 2.3.** Protocol for serial dehydration and impregnation of nerve sections.

| ***Reagents*** | |
|---|---|
| Epoxy resin (Durcupan A) | 10ml |
| Dodecenyl succinic anhydrate (DDSA) (Durcupan B) | 10ml |
| Di-*n*-butyl phthalate | 0.25ml |
| Methyl nadic anhydrate (MNA) | 0.2ml |
| BDMA | 0.5ml |
| ***Method*** | |
| Measure epoxy resin and DDSA into a disposable container using syringes and stir well with a glass rod. Measure out di-*n*-butyl phthalate with a syringe, add resin to the mixture and stir again. Use a syringe to measure out methyl nadic anhydrate and BDMA, add to the mixture and stir very thoroughly for 5 minutes. | |

**Box 2.4.** Preparation of Epoxy Resin

Whilst size and shape of myelinated nerve fibres are most accurately assessed using electron micrographs (Zimmerman et al., 1980), electron microscopy techniques are expensive, time-consuming and prone to sampling errors precluding accurate counting (Dyck et al., 1968). For these reasons light microscopy is more commonly used for nerve morphometry. A previous direct comparison of nerve fibre number and area evaluation on adjacent thin (0.1μm) and semi-thin (0.75μm) sections revealed that semi-thin sections produced measurements very similar to those obtained using electron microscopy (Zimmerman et al., 1980). Error in evaluation of semi-thin sections was greatest for small fibres, in which diameters were overestimated and myelin appeared too thick. The use of semi-thin sections of nerve fixed in aldehyde and osmium tetroxide and embedded into epoxy has now become the standard method for morphometric nerve studies, and was the method employed for the current study. This method precludes evaluation of unmyelinated fibres (Dyck, 1975). Previous research has shown that unmyelinated fibres constitute 18% or less of the total axonal regenerate population by five weeks following nerve repair (Spector et al., 1991), this being sufficient time for the axon population to achieve steady state compared with control (Spector et al., 1991). The unmyelinated fibres consist of sensory, autonomic and secretomotor fibres (Spector et al., 1991; Spector, 1997) and do not contribute significantly to motor activity (Spector et al., 1991).

## 2.9. Nerve Sectioning and Staining

For this study, 0.5μm sections were used for quantitative nerve analysis. This is thinner than used elsewhere for nerve morphometry, the advantages of which are improved

definition of microscopic images and reduced overestimation of fibre area and diameter caused by oblique sectioning of some nerve fibres (Dyck et al., 1968)

An ultramicrotome (UltraCut, Reichert-Jung, Germany) and glass knife were used to cut 0.5µm sections of the nerve samples embedded within the resin blocks, collected in a bath of 10% acetone mounted on the glass knife (Figure 2.9). These sections were transferred onto a drop of 10% acetone on a clean glass slide, stretched using a chloroform-soaked wooden stick and the slides gently dried using a methanol lantern. These were next stained with thionin (myelin stain) and counterstained with acridine orange (Box 2.7) (Sievers, 1971): a few drops of thionin were placed over the samples and the slide gently heated over a methanol lantern for 10 seconds; excess stain was washed off with distilled water and the process repeated for acridine orange. The slide was dried once more over a methanol lantern and a cover slip mounted over the sections using epoxy resin, leaving the sections ready for microscopic examination.



**Figure 2.9.** (a) Nerve sections embedded in epoxy resin. (b) Ultramicrotome used for cutting semi-thin sections. (c) Semi-thin sections were cut using a glass knife and (d) floated onto a bath of 10% acetone.

# CHAPTER 3

# RESULTS:

# DESCRIPTION & VALIDATION OF

# NERVE MORPHOMETRY METHOD

## 3.1. Introduction

In view of the extensive requirement for morphometric quantification of nerve biopsy specimens for this work, errors inherent to quantification by sampling of portions of biopsy specimens, and the hardware, software and/or economic difficulties in implementing existing methods of nerve morphometry (see Section xxxxx), it was felt necessary to modify significantly those methods currently in use for the purpose of this work. This chapter describes the method used and its validation.

## 3.2. Development of a Combined Automated and Semi-Automated Method of Nerve Morphometry.

Several existing commercially available (e.g. Image-Pro Plus, Media Cybernetics UK) and public domain (e.g. NIH Image, ImageJ) image analysis software packages permit rapid morphometric analysis of 'particles' within a digital image imported from a wide range of sources. ImageJ (Rasband, 1997-2005) was selected for its ready availability (the software can be freely downloaded from the internet), extensive use in previous medical and scientific research (Bailey et al., 2002; Gering and Atkinson, 2004; Girish and Vijayalakshmi, 2004; Brown et al., 2005; Cho and Daniel, 2005; Lau et al., 2005; Williams et al., 2005), accessible user interface and versatility for future extended applications through the use of plug-ins.

The intended use of ImageJ for 'particle analysis', the evaluation of any particles (defined by the software as any structure with a continuous outline) within an image, is as follows: the image is opened and converted to 8-bit (greyscale). It is then 'Thresholded', meaning a grey range is selected within which all greys are converted to black, and outside which all greys are converted to white, creating a binary image in which particles of interest are represented in black and artefact or irrelevant structures are eliminated into a white background. This image is then suitable for particle analysis, in which all black regions are detected and variables such as area, perimeter and circularity are calculated for each particle. A total particle count is also provided.

This approach works best for images containing non-overlapping particles exhibiting high contrast from a featureless background, but nerve biopsy images do not exhibit these characteristics. Nerve fibres may be touching, axons and background artefact may exhibit equally high contrast from their surroundings, and staining intensity may be heterogeneous. Moreover, since the software defines a particle as any structure with a continuous outline, where there are rings within rings (such as nerve fibres within a fascicle, or an axon within a nerve fibre) only the outermost ring is analysed. To analyse structures within a ring it is necessary to interrupt the ring by at least one pixel using drawing tools incorporated within the software.

Preliminary investigation revealed that histomorphometry of a raw nerve section using ImageJ in this way provided unsatisfactory results (Figure 3.1). The use of a high detection threshold (Figure 3.1a) resulted in many missed detections without eliminating

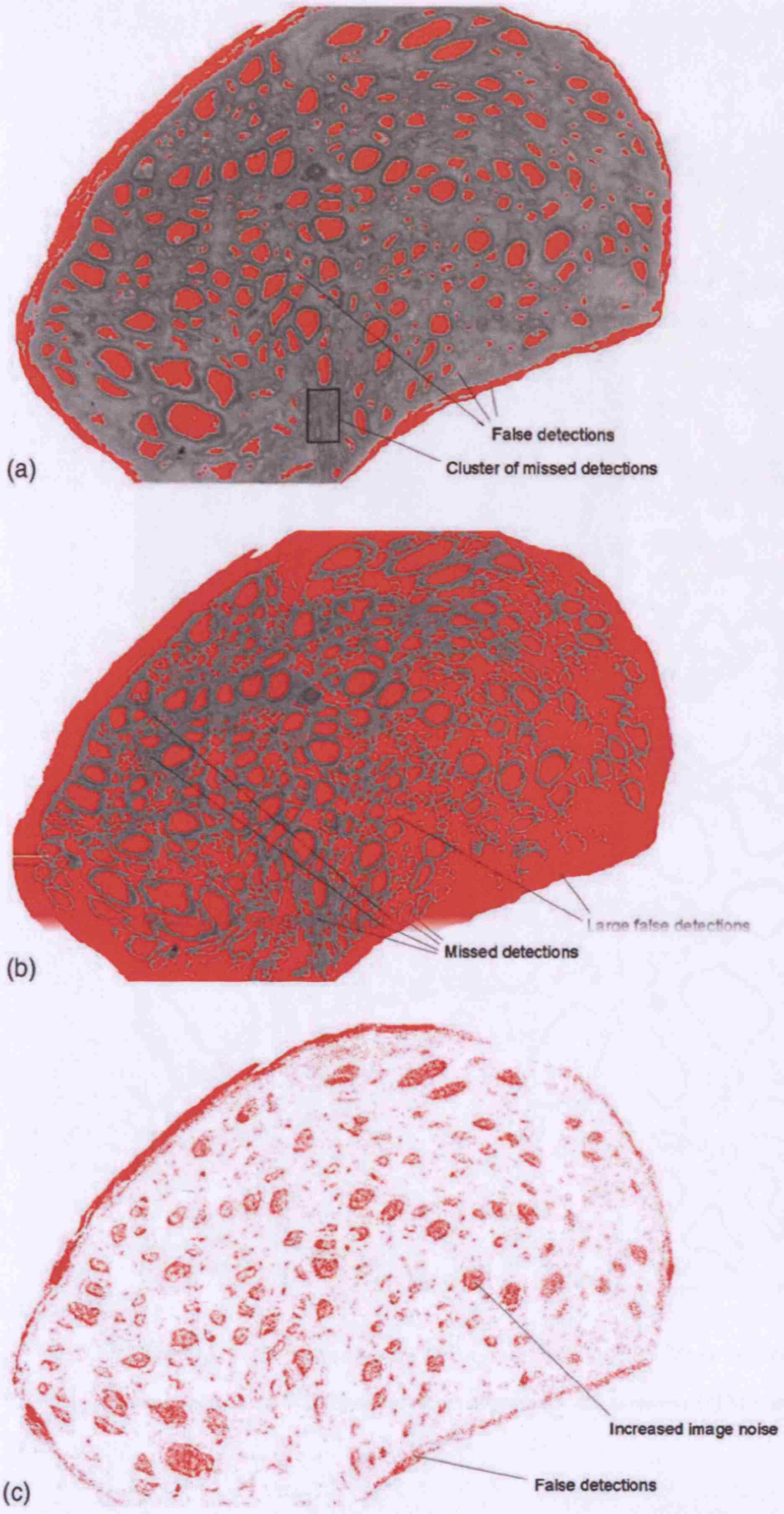



**Figure 3.1.** Particle detection within a typical nerve biopsy image using ImageJ and varying the detectio thresholds. The background image is shown in greyscale and software-detected particles are shown in red. Threshold adjustment produces either (a) many missed detections, or (b) many false detections, but in neither case is either type of error eliminated. Prior electronic background subtraction (c) incompletely reduced artefact selection and increased image noise.

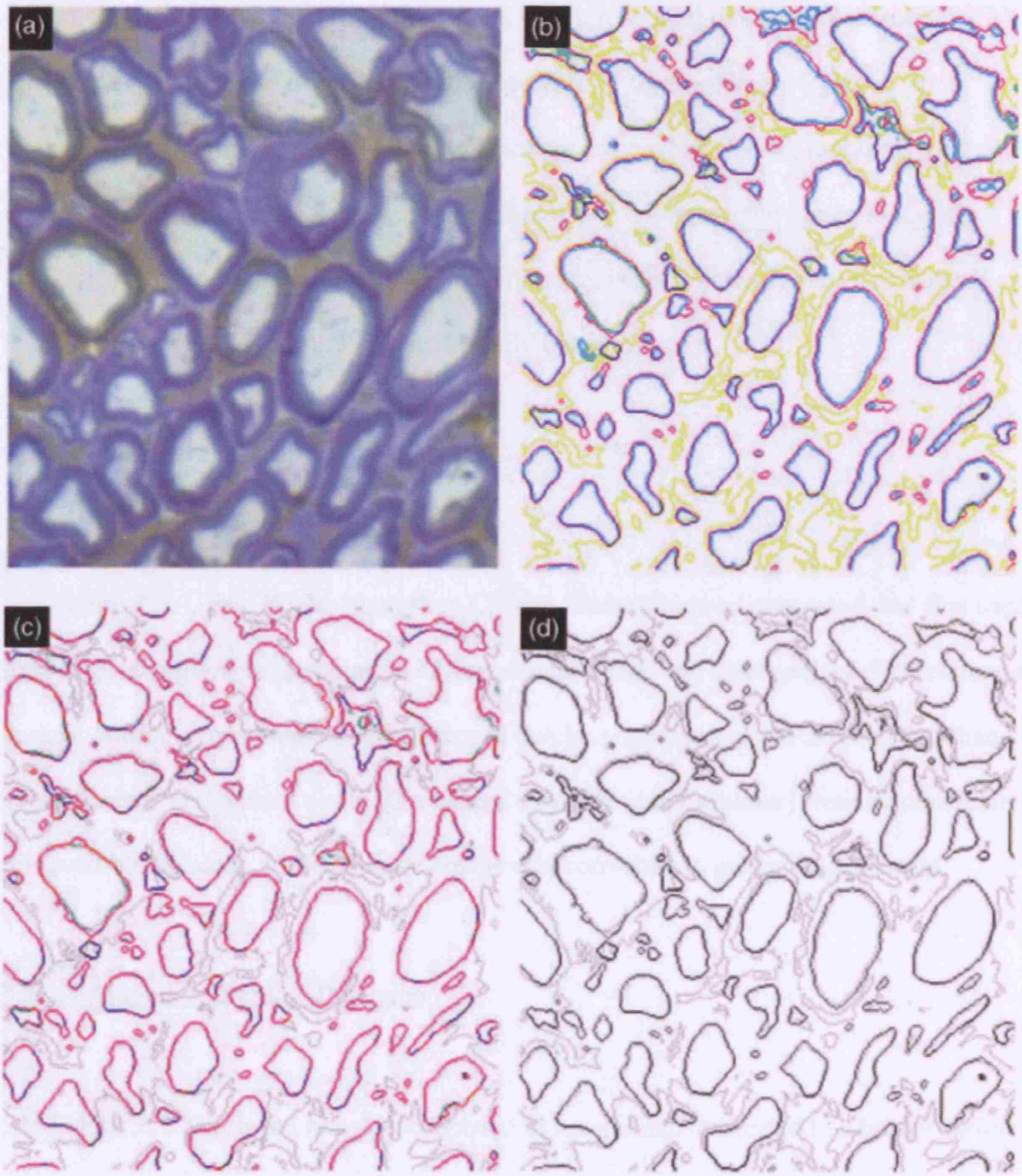



**Figure 3.2.** Image preparation. (a) The original image has (b) a 'Trace Contour' command applied followed by (c) subtraction of non-Axon outlines rendered in yellow and turquoise. (d) The image is finally saved in greyscale.

false detections, whilst the use of a low detection threshold produced many false detections without eliminating missed detections (Figure 3.1b). The software permits digital background subtraction of the image, which renders the background more homogeneous aiming to improve particle detection by thresholding. However, prior background subtraction typically increased image noise whilst failing to improve either type of error (Figure 3.1c). It was therefore necessary to prepare the image first using a different software package. Details are described by Urso-Baiarda et al. (Urso-Baiarda and Grobbelaar, 2006) but are repeated briefly below.

### 3.2.1. Image Preparation

See Figure 3.2. Using Adobe Photoshop CS, the 'Trace Contour' command was first used to provide an outline representation of the original image and levels adjusted to produce a darkest outline corresponding to the internal myelin edge (to measure axons rather than nerve fibres). Non-axonal outlines (rendered in yellow and turquoise) were automatically removed by replacing with white, the image was converted to greyscale and saved.

### 3.2.2. Automated Particle Detection

See Figure 3.3. The saved image was opened in ImageJ and converted to a binary outline image by threshold adjustment. This produced an image of individual axon outlines, as well as outlines of interstitial artefact and of the entire fascicle(s).

Automated image analysis was then run and set to output 'Masks', producing an image in which axons were represented as solid black areas. Error from both missed detections and interstitial and external artefact were also apparent. Linear artefact could be rapidly removed using the 'Smooth' command to blur it followed by repeating the threshold adjustment to filter out blurred lines. This image was saved.

### 3.2.3. Semi-Automated Image Analysis

The image obtained thus far still contained solid areas of interstitial and other artefact, as well as a few axons that had not been detected. Such errors were corrected by semi-automated analysis: the most recent binary image was opened as a semi-opaque layer over the original nerve image (Figure 3.4a) in Photoshop CS, permitting visual assessment of the accuracy of automated analysis. The image was systematically inspected frame by frame and any errors manually corrected onto the binary layer using standard drawing tools (Figure 3.4b). When finished, the binary layer opacity was returned to 100% and the corrected binary image was saved (Figure 3.4c).

### 3.2.4. Calibration

Calibration was performed once using a graticule image (0.01mm, Wild, Switzerland) captured under light microscopy using the same magnification as the nerve biopsy specimens. Calibration settings were input using the Set Scale command in ImageJ. The pixel size corresponded to 0.166μm.

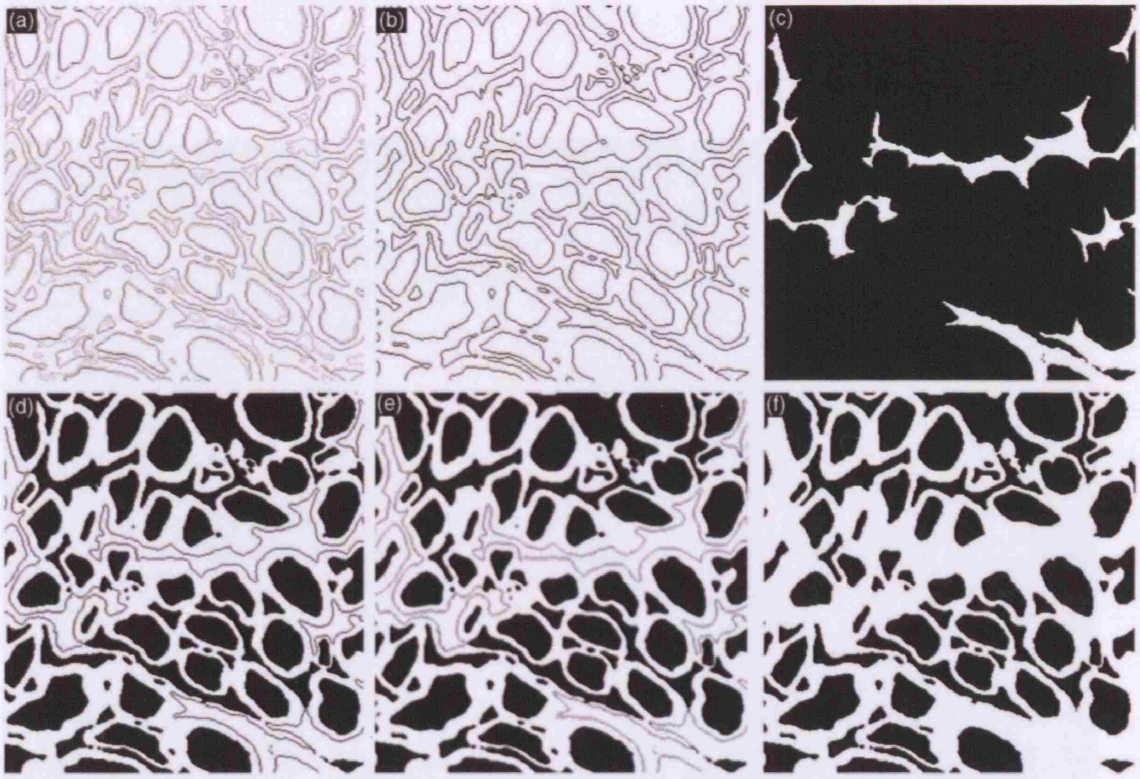



**Figure 3.3.** Automated particle detection using ImageJ. (a) The original greyscale image is opened and (b) converted to a binary image by thresholding. (c) In order to prevent recognition of a single large particle, (d) the external fascicular and confluent myelin outlines must be interrupted. Linear artefact is then removed by (e) performing the smooth command to blur it, followed by (f) repeating the threshold command.

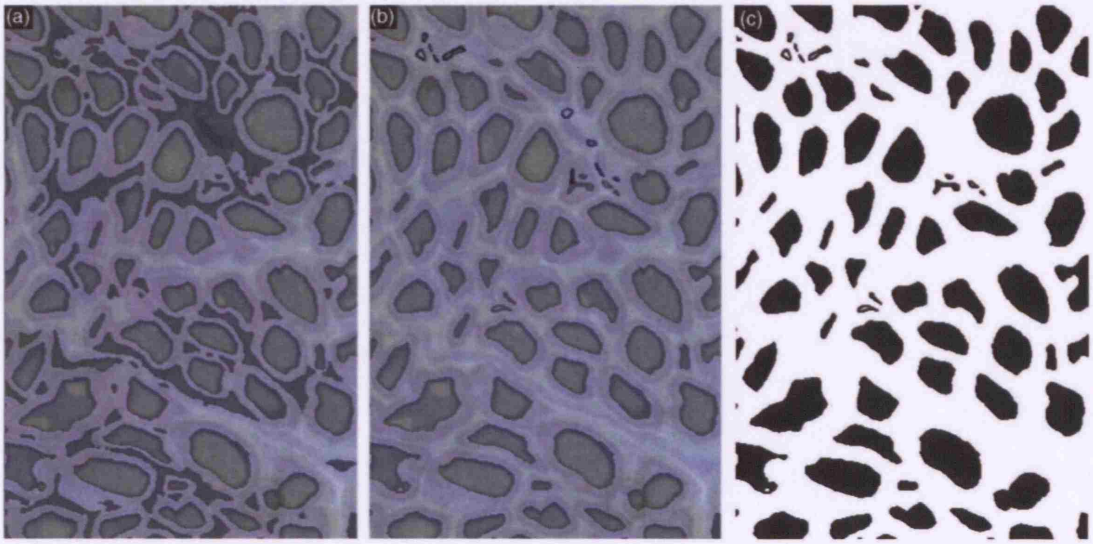



**Figure 3.4.** Semi-automated particle analysis. (a) Errors of missed detection and false detection, usually of interstitial artefact, are apparent by overlaying the last saved image as a semi-opaque layer over the original image. (b) Errors are then systematically removed frame-by-frame, (c) resulting in a corrected binary image representing axons.

**Figure 3.5.** Nerve morphometry using a standard semi-automated digitiser pen method.

### 3.2.5. Data Output

Finally, the last saved nerve image was reopened in ImageJ for final particle analysis. The data obtained could be exported to other statistics or spreadsheet software for further analysis.

## 3.3. Standard Semi-Automated Nerve Morphomety

The semi-automated method of nerve morphometry has been described previously (Koller et al., 1997; Nehrer-Tairych et al., 2000). Briefly, the image was enlarged by 500% and printed on 42-inch roll paper using a Hewlett Packard DesignJet 500PS large format printer. Axon counting and area measurement were performed by systematically tracing each Axon outline using a digitising tablet and pen (GTCO Calcomp Inc, Scottsdale, AZ) coupled to a personal computer (Figure 3.5). Data was automatically imported into a spreadsheet package (Excel, Microsoft Corp.) for further analysis.

## 3.4. Comparison of Methods

### 3.4.1 Animal and Tissue Preparation

Two biopsies of each of the motor nerve to rectus femoris, marginal mandibular branch of the facial nerve and ventral ramus of buccal branch of the facial nerve were obtained from female New Zealand White Rabbits (2500-3500g).

Anaesthesia was induced with 0.7ml intramuscular Hypnorm (Janssen, Oxford; fentanyl citrate 0.15mg/ml; fluanisone 10mg/ml) followed by 0.4ml intravenous diazepam, and maintenance anaesthesia was achieved using intravenous fentanyl and fluanisone ('Hypnorm') as required. The nerves were dissected and a short sample excised distally. Details of preparation and surgical technique are given in Chapter 2 (Section 2.4). Nerve biopsy specimens were processed immediately for histological and quantitative morphometric analysis as described in Chapter 2 (Sections 2.9 and 2.10).

### 3.4.2 Image Acquisition

Overlapping colour digital photographs of each section were taken at x40 magnification using a microscope (Zeiss Axiophot) with a mounted digital camera (Leica) and imported into a personal computer using Adobe Photoshop CS (Adobe Systems Inc., USA). The final image was automatically reconstituted from its component overlapping images using the 'Photomerge' command (File>Automate>Photomerge).

### 3.4.3 Image Analysis

Image analysis was performed both by traditional semi-automated morphometry and using combined fully- and semi-automated morphometry.

### 3.4.4 Statistical Methods

Results obtained using each method were compared for axon count, axon area and analysis time per nerve section. Each method was compared using the independent samples t-test for mean axon area, Pearson correlation for axon counts and Wilcoxon signed ranks test for axon counts and time taken to perform image analysis. Statistical tests were performed using Statistics Package for the Social Sciences (SPSS) version 11.0.4 for Mac OS X. The results are summarised in Table 3.1.

## 3.5. Results

### 3.5.1. Axon Counts

Axon counts using the combined fully- and semi-automated method ranged from 98.7-106.1% of the axon count using the semi-automated method alone, with a Pearson correlation of 0.998 (Figure 3.6). There was no significant difference between axon counts performed by either method ($p=0.600$).

### 3.5.2. Axon Area

Mean axon areas for each nerve using both the combined and semi-automated methods are summarised in Figure 3.7. There was no significant difference in mean axon area obtained using either method for any nerve ($p=0.211$-$0.928$). Moreover, each nerve

displayed a characteristic axon size frequency distribution that exhibited good correlation between methods (Figure 3.8, Pearson correlations of 0.860, 0.958, 0.884, 0.955, 0.997 and 0.995, for nerves 1-6 respectively, $p<0.001$ each).

### 3.5.3. Analysis Time

The semi-automated method took between 2 and 3 times longer to perform than the combined method, which was statistically significant ($p=0.028$, Table 3.6). There appeared to be an inverse relationship between axon number and ratio of combined:semi-automated analysis time, suggesting greatest benefit from the new method in terms of analysis time for nerves containing most axons (Pearson correlation –0.734), although this relationship was not statistically significant ($p=0.097$).

**Table 3.1.** Axon number, mean axon area and analysis time using traditional semi-automated and combined automated & semi-automated methods (Rectus = motor nerve to rectus femoris; MM = marginal mandibular branch of facial nerve; VRB = ventral ramus of buccal branch of facial nerve.)

| *Nerve* | | **Axon no.** | | **Mean Axon area /μm² (SD)** | | **Analysis Time /mins** | |
|---|---|---|---|---|---|---|---|
| | | *Semi-Automated Method* | *Combined Method (% Semi-Automated)* | *Semi-Automated Method* | *Combined Method (% Semi-Automated)* | *Semi-Automated Method* | *Combined Method (% Semi-Automated)* |
| ***Rectus*** | 1 | 790 | 780 (98.7%) | 56.1 (54.8) | 55.3 (51.2) | 140 | 52 (37.1%) |
| | 2 | 719 | 763 (106.1%) | 32.2 (30.3%) | 32.4 (32.3) | 121 | 43 (35.5%) |
| **MM** | 3 | 315 | 334 (106.0%) | 19.3 (12.8) | 17.5 (12.4) | 55 | 23 (41.8%) |
| | 4 | 614 | 615 (100.2%) | 13.9 (9.3) | 17.2 (11.4) | 88 | 39 (44.3%) |
| **VRB** | 5 | 4190 | 3991 (95.3%) | 20.4 (12.8) | 20.2 (12.9) | 1140 | 395 (34.6%) |
| | 6 | 5301 | 5513 (104.0%) | 16.3 (12.0) | 16.6 (12.1) | 1462 | 480 (32.8%) |

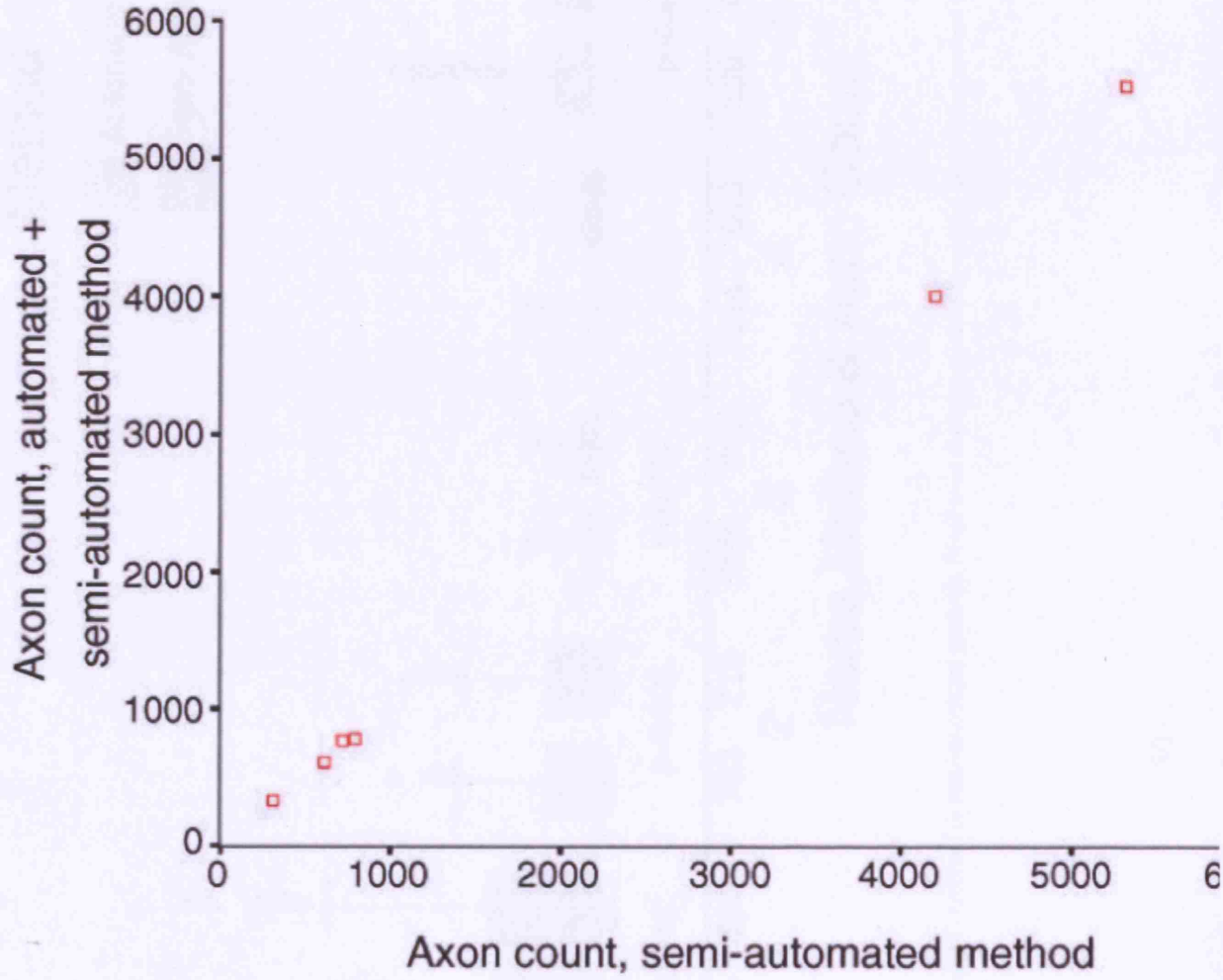



**Figure 3.6.** Correlation between axon counts obtained using combined or semi-automated morphometry (Pearson correlation = 0.998, $p<0.01$).

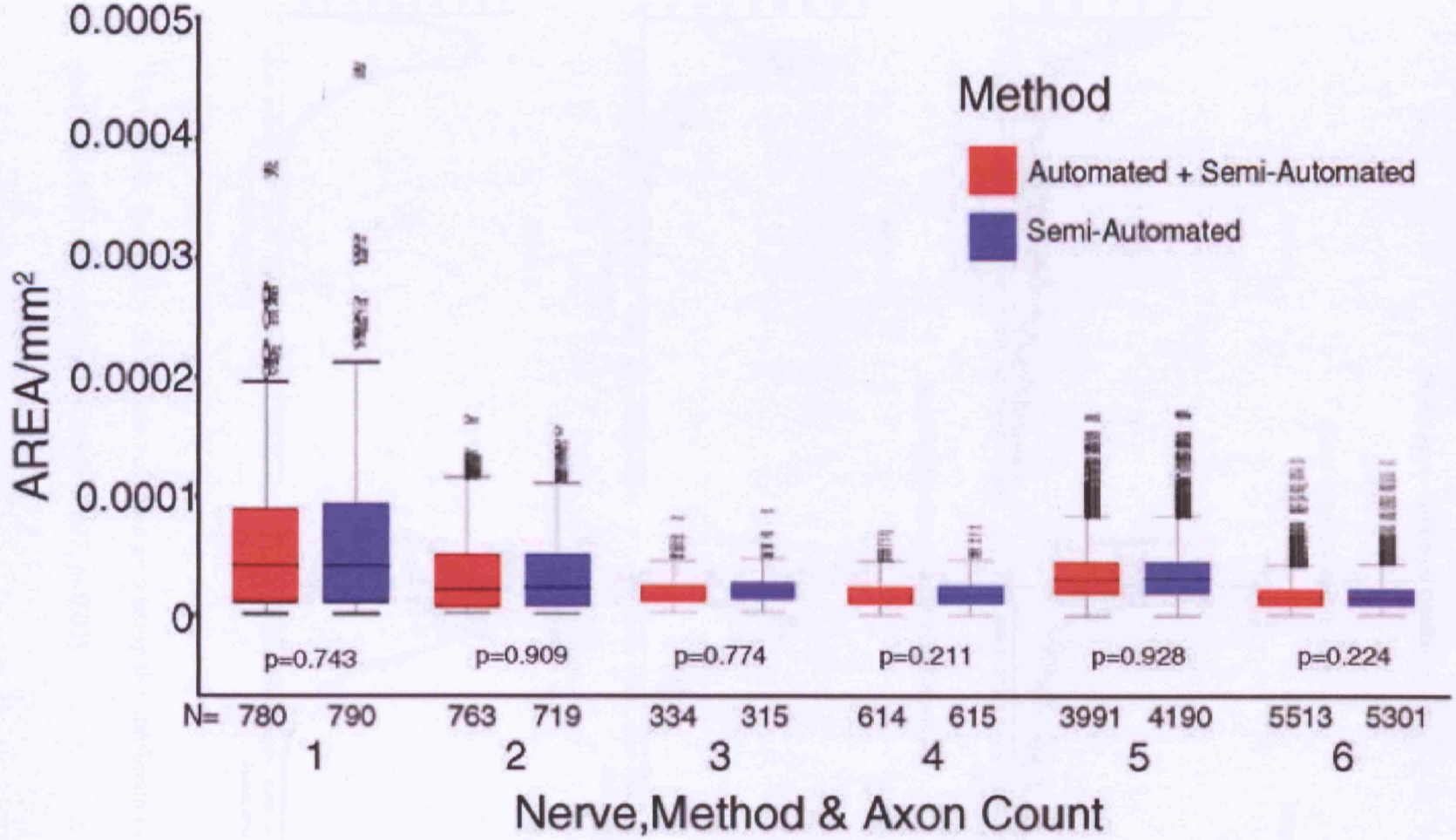



**Figure 3.7.** Axon area assessed using the combined or semi-automated methods. Mean axon area was statistically similar using either method for all nerves (p=0.211-0.928).

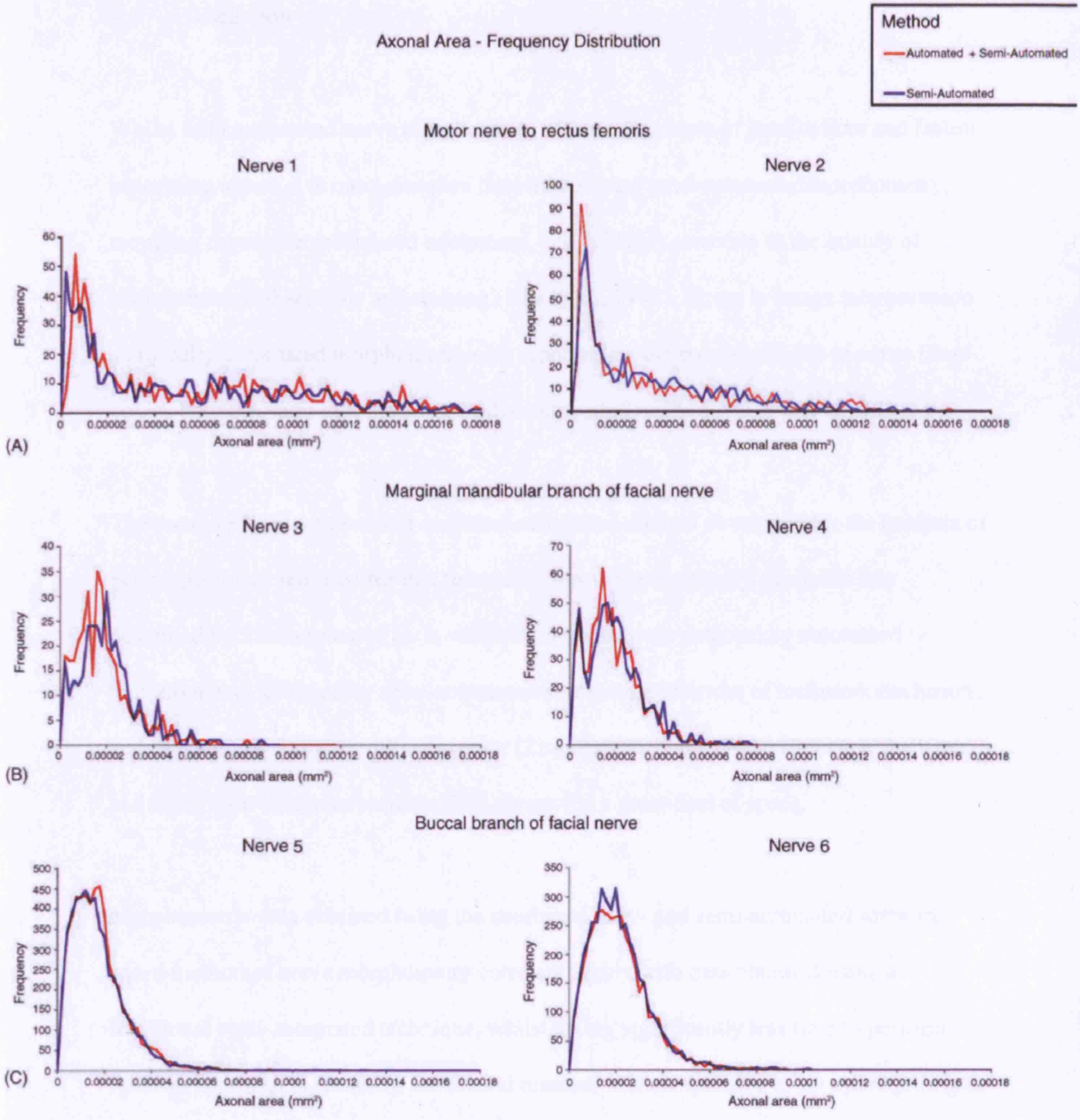



**Figure 3.8.** Frequency distribution of axon area using the combined or semi-automated methods (Pearson correlation = 0.860-0.997, $p<0.01$).

## 3.6. Discussion

Whilst fully automated nerve morphometry offers advantages of least tedium and fastest processing speed, it is more complex than manual and semi-automated morphometry, requiring expensive specialised equipment, and is highly sensitive to the quality of histopathological sections and staining (Vita et al., 1992). Errors in image interpretation using fully-automated morphometry may amount to a difference of 8.3% in nerve fibre counts between semi-automated and fully-automated methods (Usson et al., 1991).

The combined fully-automated and semi-automated method developed for the analysis of nerve specimens required for this research project is conceptually similar to that described by Zimmerman et al. in which 85% axons were detected by automated morphometry, followed by semi-automated elimination of errors of inclusion, exclusion and overlap in a frame-by-frame manner (Zimmerman et al., 1980). However, the latter had significant hardware requirements occupying a great deal of space.

Morphometric data obtained using the combined fully- and semi-automated software-based method of nerve morphometry correlate highly with data obtained using a traditional semi-automated technique, whilst taking significantly less time to perform. Such an approach is attractive for several reasons. It is inexpensive to set up, requiring in addition to standard laboratory equipment only a computer running two readily available software packages, with negligible running costs. The method does not require dedicated laboratory space, and is indeed portable, allowing work to take place opportunistically

when time is available. The method is also rapid enough to avoid data sampling and provides a record of the exact outlines that have been measured for comparison with the original image, permitting subsequent critical evaluation of the quality of data obtained. If errors are noted at a later stage it is possible to correct them and repeat the entire analysis in only a few seconds.

There was a trend towards greatest timesaving benefit for the largest nerves, although this trend did not achieve statistical significance. Experience of using the technique, however, suggests that greatest timesaving for largest nerves is plausible. This is because for the combined method, the fully automated phase of nerve morphometry takes a constant amount of time to perform (regardless of nerve size), and only the semi-automated component takes an amount of time dependent on nerve size. For larger nerves with greater overall analysis times, the relative impact of the fully automated component progressively diminishes.

# CHAPTER 4

# RESULTS:

# NERVE MORPHOMETRY DATA

## 4.1. Introduction

Functional muscle transfer (FMT) combined with cross-facial nerve grafting (CFNG) is the modern gold standard treatment of chronic unilateral facial palsy. However, the few published large international clinical series suggest a highly variable clinical outcome resulting from excessively weak to unduly forceful FMTs. Previous research using the rabbit rectus femoris FMT model showed that ultimate force production correlates with the magnitude of donor motor input (MacQuillan, 2005), and on theoretical grounds FMTs are thought to be highly sensitive to random between-patient variations in motor axon load due to the relative paucity of reinnervating motor axons and a postulated tendency to form maximally-sized motor units (Urso-Baiarda et al., 2006). These phenomena could contribute to the variable clinical outcome of FMT with CFNG, the corollary being that outcome could be regulated by precisely controlling FMT neurotisation.

Such precise control is not possible through motor nerve branch selection. Donor nerve branches to be used for FMT reinnervation are selected based on the effect that their activity has on native muscle. In other words, a FMT inset in such a way as to elevate the corner of the mouth on shortening must be reinnervated by a donor facial nerve branch whose stimulation elevates the contralateral corner of the mouth, irrespective of the size of that branch. Indeed there may be large size discrepancies in functionally correct facial nerve branches between different individuals as a result of considerable variability in facial nerve anatomy at that location (Kwak et al., 2004). It is, in an case, impossible to

know the axon content of a nerve branch intraoperatively since quantification is very time consuming (Urso-Baiarda and Grobbelaar, 2006). Even if such quantification were possible at the time of surgery, neural escape at proximal and distal neurorrhaphy sites and failure of axons to cross the entire length of the nerve graft are random processes, so axon supply may be dissimilar to axon delivery to the FMT.

Since it therefore appears impossible to regulate FMT neurotisation by means of motor nerve branch selection, attention has turned to the possibility of using the interposed nerve graft to manipulate axon transmission from donor nerve to FMT. It was hypothesised that an interposed nerve graft of low axon-transmitting capacity might result in impaired and graft-dependent neurotisation both of the graft and of any coapted end organ, whereas a high-capacity graft permits high fidelity (and therefore motor nerve-dependent) transmission of axons to FMT. Assuming a relationship between FMT reinnervation, force production and clinical outcome, the latter is likely to produce variable end organ function and clinical outcome from variable motor input, whereas the former should produce more constant end organ function and clinical outcome irrespective of diverse motor input magnitude (so long as the capacity for axon transmission is exceeded by donor motor axon availability).

## 4.2. Aims

Light microscopic examination and morphometric quantification of nerve biopsies were performed with the following aims:

To validate group methodology as described in Chapter 2. Specifically, to establish a) that the marginal mandibular (MM) and ventral ramus of buccal (VRB) branches of the facial nerve provided motor inputs of significantly different magnitude, and b) that the saphenous (S) and medial saphenous (SB) nerves possessed significantly different cross sectional areas and numbers of myelinated fibres, therefore potentially yielding nerve grafts possessing different numbers of Schwann cells.

To examine and quantify patterns of axon regeneration within nerve grafts at three (where possible) and nine month time points, and within the nerve to rectus femoris at nine months, in order to determine any effect of motor nerve input and nerve graft properties on neurotisation of the nerve graft itself and the rectus nerve distal to it.

## 4.3. Methods

Nerve biopsies were taken for morphometric analysis at three separate time points. At 0 months, during first or one-stage procedures, a minimum of 6 rectus nerve, motor nerve (VRB or MM) and nerve graft (S or SB) biopsies were taken. At 3 months, during second stage procedures (in those animals undergoing two-stage surgery), the distal end of nerve grafts were biopsied. Finally in all animals at 9 months, during final procedures, nerve graft and rectus nerve were biopsied (Table 4.1).

Biopsies were processed as described in Chapter 2 (Section 2.9) and underwent morphometric analysis as described in Chapter 3 (Section 2.4). Statistical comparison of

mean axon area and mean axon count between groups was performed by means of the independent samples t-test after confirming normal distribution of study population data. If data failed Levene's test for equality of variance then statistical comparisons were made using the Mann-Whitney Rank Sum test. Mean nerve graft cross-sectional areas were calculated as the total area of individual fascicles, tissue between fascicles being excluded, and were also compared with the independent samples t-test or Mann-Whitney Rank Sum test. Statistical tests were performed using Statistics Package for the Social Sciences (SPSS) version 11.0.4 for Mac OS X.

## 4.4. Terminology

Since biopsies of different nerve types originating from different study groups were taken at different time points, consistent terminology for describing nerve data was defined to maintain economy of language without compromising clarity. Up to three components could be designated for any nerve biopsy: the identity of the biopsied nerve itself; the experimental time point at which the biopsy was taken; and the study group to which the biopsied nerve belongs. This information was abbreviated as shown in Figure 4.1.

Using this terminology, $R_9^{VRBS1}$ refers to the rectus nerve (R) biopsied at 9 months ($_9$) from an animal having undergone one stage surgery ($^1$) using a ventral ramus of buccal ($^{VRB}$) motor nerve and a saphenous ($^S$) nerve graft. Part of this information was omitted in

**Table 4.1.** Nerve biopsies harvested and quantified at the three experimental time points.

| Time (months) | Motor Nerve (VRB or MM) | Nerve Graft (S or SB) | Rectus nerve |
|---|---|---|---|
| 0 | ✔ | ✔ | ✔ |
| 3 | | ✔ (2-stage groups only) | |
| 9 | | ✔ | ✔ |

$$\text{NERVE}^{\text{study group}}_{\text{time point}}$$

| Nerve Identity | | Time point (subscript) | | Study group (superscript) | |
|---|---|---|---|---|---|
| MM | marginal mandibular branch of facial nerve | 0 | 0 month time point (first or one-stage surgery) | $\text{VRB}_{\text{or}}^{\text{MM}}$ | donor nerve (marginal mandibular/ ventral ramus of buccal) |
| VRB | ventral ramus of buccal branch of facial nerve | 3 | 3 month time point (second stage surgery) | $\text{S}_{\text{or}}^{\text{SB}}$ | nerve graft (saphenous or medial saphenous branch) |
| S | saphenous nerve graft | 9 | 9 month time point (harvesting procedure) | $1_{\text{or}}^{2}$ | one or two stage surgery |
| SB | medial saphenous branch nerve graft | | | | |
| R | rectus nerve | | | | |

**Figure 4.1.** Terminology for the description of nerve biopsies.

instances where it was redundant. For instance, any nerve graft biopsy taken at 3 months must have arisen from study groups undergoing two-stage surgery (since no procedure took place at 3 months in animals undergoing one-stage surgery), and the biopsy itself indicated the type of nerve graft used for FMT reinnervation. Thus $SB_3^{MM}$ was sufficient to identify a medial saphenous nerve graft biopsy taken at 3 months from an animal undergoing two-stage surgery using the marginal mandibular motor nerve and the medial saphenous nerve graft. Similarly, control biopsies taken during first or one-stage surgery were unaffected by the future study group of the animal, which was therefore not noted. Thus $VRB_0$ refers to a biopsy of the ventral ramus of buccal branch of the facial nerve taken during first or one-stage surgery.

## 4.5. Control Nerve Biopsies (0 months)

Data are summarised in Table 4.2.

### 4.5.1. Motor nerves

$MM_0$ nerves contained on average 505.00 (SD=138.28) axons of mean area 18.01$\mu m^2$ (SD=2.63), whereas $VRB_0$ contained on average 3853.83 axons (SD=970.21) of mean area 18.93$\mu m^2$ (SD=2.42). The difference in mean axon count but not in mean axon area between $MM_0$ and $VRB_0$ nerves was statistically significant ($p<0.001$ and $p=0.529$ respectively, t-test).

4.5.2. Nerve grafts

$S_0$ biopsies possessed 2552.86 (SD=620.59) axons with a mean area of 8.04$\mu m^2$ (SD=1.32). $SB_0$ biopsies contained 1045.29 (SD=316.41) axons of mean area 6.17$\mu m^2$ (SD=1.41). The difference between $S_0$ and $SB_0$ in both mean axon count and mean axon area was statistically significant ($p<0.001$ and $p=0.025$ respectively, t-test). There was also a significant greater total cross sectional fascicular area in S (91056.17$\mu m^2$, SD=28676.80) than SB (31616.63$\mu m^2$, SD=19825.79) nerve grafts ($p=0.001$, t-test).

4.5.3. Rectus nerve

$R_0$ contained on average 887.67 (SD=116.52) axons. Mean axon area, at 41.10$\mu m^2$ (SD=9.80), was considerably larger than that of any other motor nerves or nerve grafts used in this study ($p \leq 0.002$ each, t-test). $R_0$ axon count was statistically dissimilar to all nerves used in this study except for $SB_0$ ($p=0.256$, t-test), $MM_0$ containing fewer and $S_0$ and $VRB_0$ containing greater axons ($p \leq 0.001$ each, t-test).

## 4.6. Second Stage Nerve Biopsies (3 months)

There was the opportunity to biopsy the distal end of nerve grafts at 3 months during the second stage procedure in animals undergoing two-stage surgery, prior to making the distal coaptation with the FMT. Data are summarised in Table 4.3.

**Table 4.2.** Mean axon count & mean of mean axon area for motor nerves ($MM_0$ & $VRB_0$), nerve grafts at the time of harvest ($SB_0$ & $S_0$) and rectus nerve at the time of functional muscle transfer ($R_0$).

| Nerve | n = | Axon count (SD) | Axon area/um$^2$ (SD) | Total fascicular area/ um$^2$ (SD) |
|---|---|---|---|---|
| $MM_0$ | 7 | 505.00 (138.28) | 18.01 (2.63) | |
| $VRB_0$ | 6 | 3853.83 (970.21) | 18.93 (2.42) | |
| $SB_0$ | 7 | 1045.29 (316.41) | 6.17 (1.41) | 31616.63 (19825.79) |
| $S_0$ | 7 | 2552.86 (620.59) | 8.04 (1.32) | 91056.17 (28676.80) |
| $R_0$ | 6 | 887.67 (116.52) | 41.10 (9.80) | |

**Table 4.3.** Mean axon counts and mean of mean axon areas of nerve grafts at 3 months (statistical values calculated using Mann-Whitney rank sum test).

| Nerve | n= | Axon count (SD) | p= | | | | Axon area /um$^2$ (SD) | p= | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $SB_3^{MM}$ | 5 | 906.00 (285.37) | 0.402 | 0.009 | 0.009 | 0.028 | 2.80 (0.74) | 0.347 | 0.117 | 0.602 | 0.347 |
| $SB_3^{VRB}$ | 5 | 1035.40 (436.77) | 0.009 | 0.028 | | | 3.30 (0.69) | 0.917 | 0.465 | | |
| $S_3^{MM}$ | 5 | 1634.20 (234.90) | | | | | 3.77 (0.94) | | | | |
| $S_3^{VRB}$ | 5 | 2452.80 (974.97) | | | | | 3.40 (1.27) | | | | |

### 4.6.1. Mean Axon Area

Mean axon area was 2.80μm$^2$ (SD=0.74) in $SB_3^{MM}$, 3.30μm$^2$ (SD=0.69) in $SB_3^{VRB}$, 3.77μm$^2$ (SD=0.94) in $S_3^{MM}$ and 3.40μm$^2$ (SD=1.27) in $S_3^{VRB}$ (Table 4.5). There was no significant difference in mean axon area between any two groups (p=0.117-0.917, Mann-Whitney test). Thus any differences in graft neurotisation between study groups were wholly attributable to differences in regenerating axon count.

### 4.6.2. Mean Axon Count

Morphometry revealed mean axon counts of 906.00 (SD=285.37) in $SB_3^{MM}$, 1035.40 (SD=436.77) in $SB_3^{VRB}$, 1634.20 (SD=234.90) in $S_3^{MM}$ and 2452.80 (SD=974.97) in $S_3^{VRB}$ (Table 4.3). In order to assess any differential effect of motor nerve input magnitude on graft neurotisation when using either S or SB grafts, comparisons of mean axon count between $SB_3^{MM}$ and $SB_3^{VRB}$ and between $S_3^{MM}$ and $S_3^{VRB}$ was made. There was no significant difference in mean axon count between $SB_3^{MM}$ and $SB_3^{VRB}$ (p=0.402, Mann-Whitney test), but significantly more axons in $S_3^{VRB}$ than $S_3^{MM}$ (p=0.028, Mann-Whitney test), indicating that motor input magnitude influenced graft neurotisation when a high- (S), but not when a low-capacity (SB), nerve graft was used.

In order to assess whether the use of a smaller SB nerve graft reduced graft neurotisation for both large (VRB) and small (MM) motor inputs, axon counts in $SB_3^{MM}$ and $S_3^{MM}$, and

in $SB_3^{VRB}$ and $S_3^{VRB}$, were compared. In both cases there was a significantly reduced axon count when using the smaller (SB) nerve graft compared with the larger (S) nerve graft ($p=0.009$ each, Mann-Whitney test), indicating that the use of an interposed low-capacity nerve graft compared with a high-capacity nerve graft reduced three month graft neurotisation regardless of the donor motor input.

### 4.6.3. Percentage Axons Crossing the Coaptation

In order to clarify any differences in axon numbers crossing the proximal nerve graft coaptation between the four groups, axon counts in the three-month nerve grafts were expressed as percentages of axon counts in the neurotising donor nerve branches. The percentage of donor nerve branch axons present in the three-month nerve graft was 26.86% in $SB_3^{VRB}$, 63.65% in $S_3^{VRB}$, 179.41% in $SB_3^{MM}$ and 323.60% in $S_3^{MM}$ (Figure 4.2). Thus there were greater or fewer axons within the nerve graft than in the original motor nerve when the MM or VRB respectively were used as the donor nerve, and for either donor nerve the proportion of axons crossing the coaptation was higher for S than SB nerve grafts.

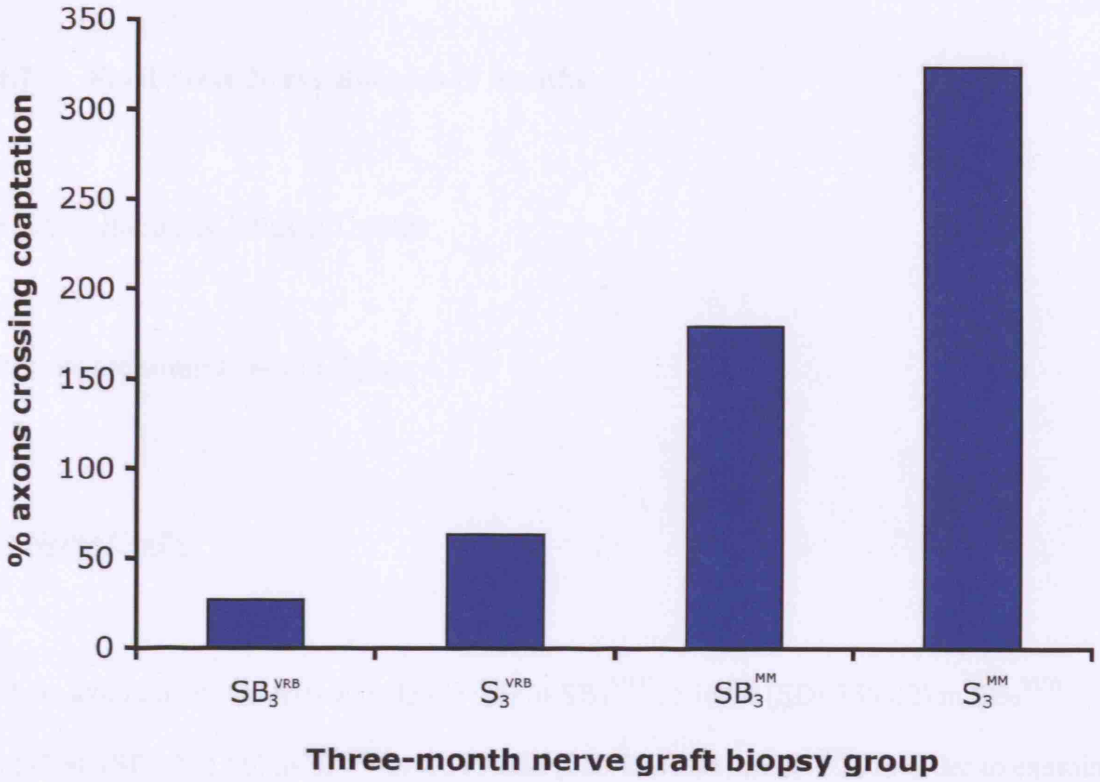



**Figure 4.2.** Percentage of axons crossing the proximal coaptation at 3 months. There were more axons within the nerve graft than in the original donor nerve when the MM was used as the donor nerve, and fewer when the VRB was used. For either donor nerve, the proportion of axons crossing the coaptation was higher for S than SB nerve grafts. S=saphenous nerve graft; SB=medial saphenous branch nerve graft; MM=marginal mandibular branch of facial nerve; VRB=ventral ramus of buccal branch of facial nerve.

## 4.7. Final Stage Nerve Biopsies (9 months)

### 4.7.1. One-Stage Surgery Groups

Results are summarised in Figure 4.3.

a) Nerve Grafts

Mean axon count was 410.00 (SD=83.34) in $SB_9^{MM1}$, 556.00 (SD=234.02) in $SB_9^{VRB1}$, 3297.40 (SD=752.16) in $S_9^{MM1}$ and 5199.80 (SD=2041.58) in $S_9^{VRB1}$. In order to examine the effect on nine-month nerve graft axon count of changes in reinnervating motor nerve for a constant nerve graft, $SB_9^{MM1}$ was compared with $SB_9^{VRB1}$ and $S_9^{MM1}$ compared with $S_9^{VRB1}$. There appeared to be a trend towards higher mean axon count in $S_9^{VRB1}$ compared with $S_9^{MM1}$, but statistically there was no significant difference in mean axon count between either these two groups (p=0.117, Mann-Whitney test) or between $SB_9^{MM1}$ and $SB_9^{VRB1}$ groups (p=0.347, Mann-Whitney test).

Similar mean axon areas were exhibited by $SB_9^{MM1}$ and $SB_9^{VRB1}$ nerve biopsies (15.59 $\mu m^2$, SD=5.50, vs.14.84 $\mu m^2$, SD=2.97 respectively; p=0.796, t-test) and by $S_9^{MM1}$ and $S_9^{VRB1}$ biopsies (4.96 $\mu m^2$, SD=1.86, vs 5.59 $\mu m^2$, SD=2.20 respectively, p=0.635, t-test). However mean axon area was significantly less for both $S_9^{MM1}$ compared with $SB_9^{MM1}$ (p=0.016, Mann-Whitney test) and for $S_9^{VRB1}$ compared with $SB_9^{VRB1}$ (p=0.001, t-test).

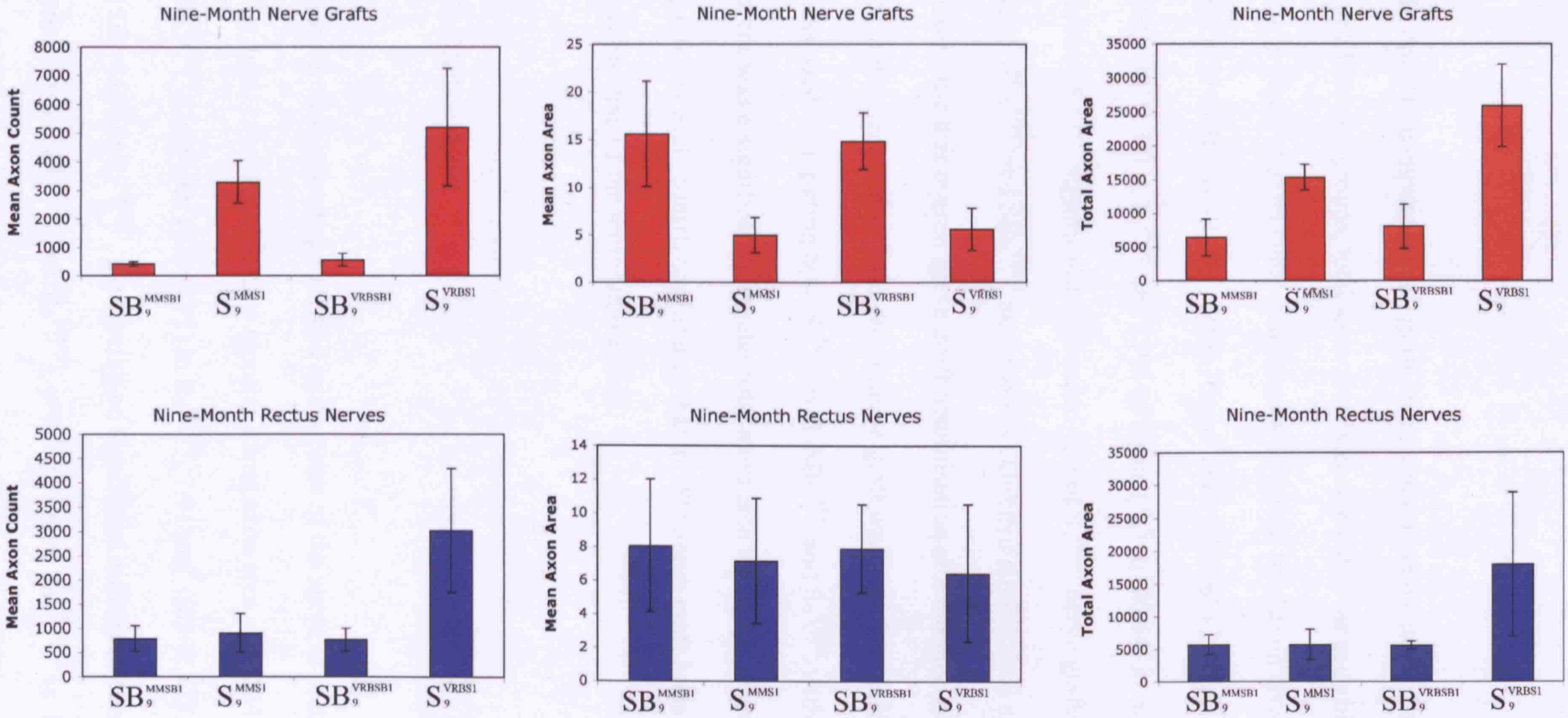



**Figure 4.3.** Nine-month nerve biopsy results for one-stage groups.

In order to compare overall graft neurotisation irrespective of differences in mean axon area between groups, total axon area was calculated for each nine-month biopsy group as the product of axon count and mean area. Mean total axon area was 6401.12 $\mu m^2$ (SD=2746.36) in $SB_9^{MM1}$, 8099.70 $\mu m^2$ (SD=3331.67) in $SB_9^{VRB1}$, 15351.73 $\mu m^2$ (SD=1879.43) in $S_9^{MM1}$ and 25954.95 $\mu m^2$ (SD=6106.44) in $S_9^{VRB1}$. Whilst total axon area of $S_9^{VRB1}$ significantly exceeded that of $S_9^{MM1}$ nerve grafts (p=0.009, Mann-Whitney test), $SB_9^{MM1}$ and $SB_9^{VRB1}$ nerve grafts exhibited similar total axon areas (p=0.405, t-test), indicating that overall nerve graft neurotisation at 9 months correlated with the magnitude of motor nerve input when the S nerve graft was used but not when the SB nerve graft was used. Comparing both $S_9^{MM1}$ with $SB_9^{MM1}$ and $S_9^{VRB1}$ with $SB_9^{VRB1}$ respectively, there was a significantly greater total axon area when the larger (S) nerve graft was used ($p<0.001$ each, t-test), confirming that the SB nerve graft impaired graft neurotisation irrespective of the motor nerve used.

b) Rectus nerves

Similar analysis was performed on biopsies of the nerve to rectus femoris (i.e. distal to the distal coaptation) at nine months. Mean axon area was 8.04 $\mu m^2$ (SD=3.96) in $R_9^{MMSB1}$, 7.15 $\mu m^2$ (SD=3.71) in $R_9^{MMS1}$, 7.91 $\mu m^2$ (SD=2.62) in $R_9^{VRBSB1}$ and 6.44 $\mu m^2$ (SD=4.09) in $R_9^{VRBS1}$, there being no significant difference between any group comparisons ($R_9^{VRBS1}$ vs. $R_9^{MMS1}$, p=0.780, t-test; $R_9^{VRBSB1}$ vs. $R_9^{MMSB1}$, p=0.952, t-test; $R_9^{MMSB1}$ vs. $R_9^{MMS1}$, p=0.602, Mann-Whitney test; $R_9^{VRBSB1}$ vs. $R_9^{VRBS1}$, p=0.518, t-test). Thus neurotisation of rectus nerves at 9 months was entirely dependent on axon count.

Mean axon count was 785.80 (SD=266.17) in $R_9^{MMSB1}$, 907.00 (SD=397.56) in $R_9^{MMS1}$, 768.00 (SD=232.89) in $R_9^{VRBSB1}$ and 3009.80 (SD=1276.39) in $R_9^{VRBS1}$. Although axon count was significantly higher in $R_9^{VRBS1}$ compared with $R_9^{MMS1}$ (p=0.008, t-test), those of $R_9^{VRBSB1}$ and $R_9^{MMSB1}$ were similar (p=0.913, t-test), indicating once again that the magnitude of motor input influenced neurotisation if the S, but not if the SB, interposed nerve graft was used. $R_9^{MMSB1}$ and $R_9^{MMS1}$ biopsies exhibited similar axon counts (p=0.587, t-test) but that of $R_9^{VRBSB1}$ was significantly lower than that of $R_9^{VRBS1}$ (p=0.005, t-test). Thus at the level of the rectus nerve, use of the SB interposed nerve graft did not impair neurotisation when a small (MM) motor input was used but did when a high-capacity (VRB) motor input was used.

These results were confirmed by comparison of total axon area for nine-month rectus nerves of each group. Total axon area was 5660.20 $\mu m^2$ (SD=1526.91) in $R_9^{MMSB1}$, 5699.32 $\mu m^2$ (SD=2354.62) in $R_9^{MMS1}$, 5591.75 $\mu m^2$ (SD=617.25) in $R_9^{VRBSB1}$ and 17910.68 $\mu m^2$ (SD=11032.29) in $R_9^{VRBS1}$. Total axon area was significantly higher in $R_9^{VRBS1}$ compared with both $R_9^{MMS1}$ (p=0.042, t-test) and $R_9^{VRBSB1}$ (p=0.016, Mann-Whitney test), other group comparisons revealing similar total axon areas ($R_9^{VRBSB1}$ vs. $R_9^{MMSB1}$, p=0.928, t-test; $R_9^{MMSB1}$ vs. $R_9^{MMS1}$, p=0.976, t-test).

4.7.2. Two-Stage Surgery Groups

Results are summarised in Figure 4.4.

a) Nerve Grafts

Results were similar to those of one-stage surgery groups. Axon count was significantly higher in $S_9^{VRB2}$ biopsies (4103.0, SD=1396.97) than in those from either $SB_9^{VRB2}$ (581.8, SD=479.21) or $S_9^{MM2}$ groups (578.6, SD=205.22) (p=0.001 each, t-test). There was no difference in axon count comparing $SB_9^{MM2}$ (523.4, SD=358.00) with either $SB_9^{VRB2}$ or $S_9^{MM2}$ (p=0.833 or p=0.773 respectively, t-test).

Mean axon area was 8.23 $\mu m^2$ (SD=3.18) in $SB_9^{MM2}$, 5.72 $\mu m^2$ (SD=2.88) in $SB_9^{VRB2}$, 9.36 $\mu m^2$ (SD=1.93) in $S_9^{MM2}$ and 6.18 $\mu m^2$ (SD=2.36) in $S_9^{VRB2}$. There was no significant difference between 3 of the 4 nerve graft biopsy group comparisons ($SB_9^{MM2}$ vs. $SB_9^{VRB2}$, p=0.227; $SB_9^{MM2}$ vs. $S_9^{MM2}$, p=0.518; $SB_9^{VRB2}$ vs. $S_9^{VRB2}$, p=0.792, all t-test), the last just achieving statistical significance ($S_9^{MM2}$ vs. $S_9^{VRB2}$, p=0.048, t-test).

Total axon area was 3647.46 $\mu m^2$ (SD=1879.21) in $SB_9^{MM2}$, 4094.67 $\mu m^2$ (SD=4302.48) in $SB_9^{VRB2}$, 5493.68 $\mu m^2$ (SD=2199.01) in $S_9^{MM2}$ and 27123.12 $\mu m^2$ (SD=18216.41) in $S_9^{VRB2}$. This was significantly higher in $S_9^{VRB2}$ biopsies than in either $S_9^{MM2}$ or $SB_9^{VRB2}$ biopsies (p=0.030 and p=0.025 respectively, t-test). There was no difference in total axon area comparing $SB_9^{MM2}$ with either $SB_9^{VRB2}$ or $S_9^{MM2}$ (p=0.837 and p=0.191 respectively,

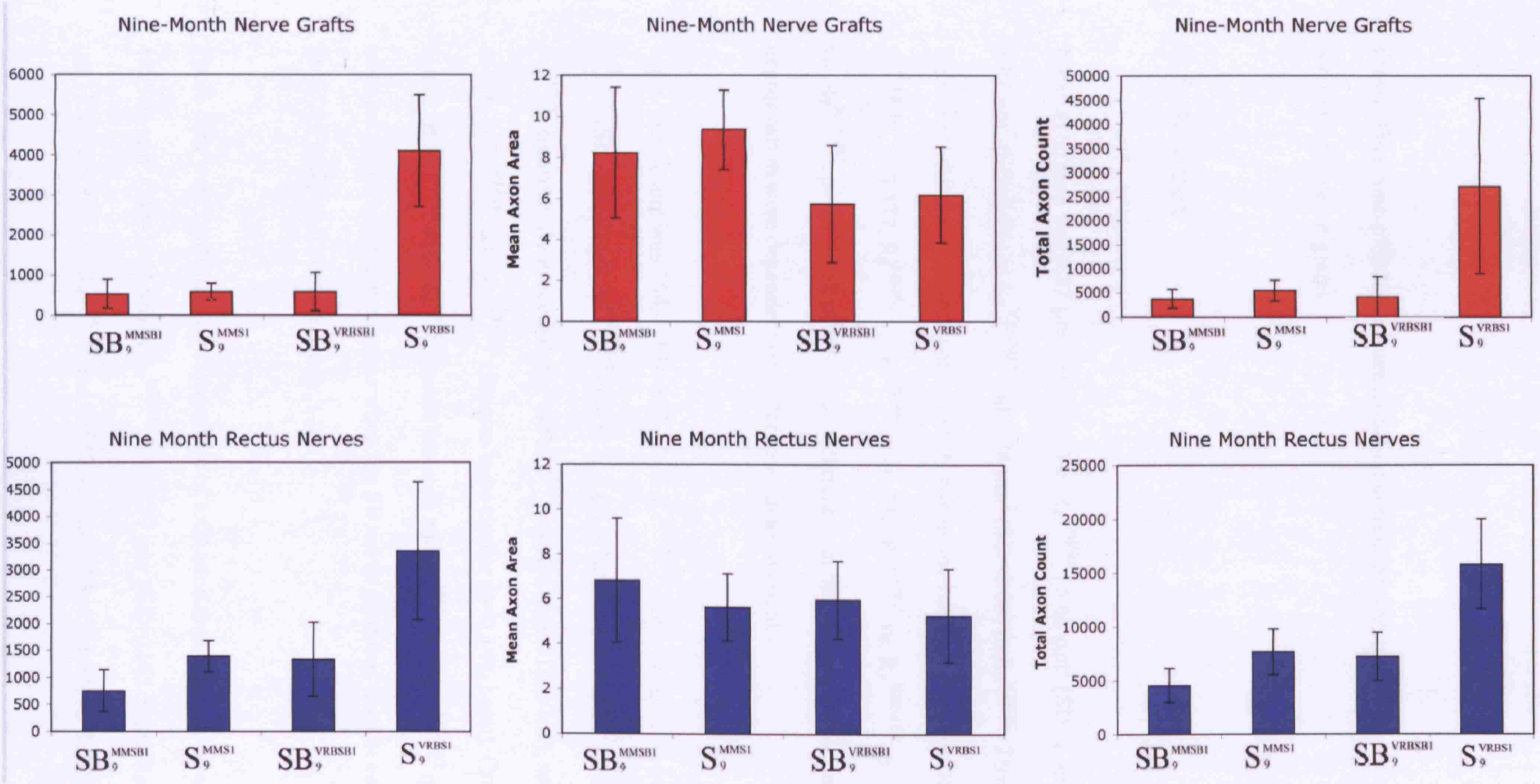



**Figure 4.4.** Nine-month nerve biopsy results for two-stage groups.

t-test). Thus nine-month graft neurotisation correlated with magnitude of motor input for S but not SB nerve grafts.

b) Rectus Nerves

Mean axon area was 6.82 $\mu m^2$ (SD=2.77) in $R_9^{MMSB2}$, 5.62 $\mu m^2$ (SD=1.49) in $R_9^{MMS2}$, 5.97 $\mu m^2$ (SD=1.73) in $R_9^{VRBSB2}$ and 5.28 $\mu m^2$ (SD=2.08) in $R_9^{VRBS2}$. There was no significant difference in mean axon area between any compared groups ($R_9^{MMSB2}$ vs. $R_9^{VRBSB2}$, p=0.577; $R_9^{MMS2}$ vs. $R_9^{VRBS2}$, p=0.771, $R_9^{VRBS2}$ vs. $R_9^{VRBSB2}$, p=0.585; $R_9^{MMS2}$ vs. $R_9^{MMSB2}$, p=0.420; all t-test). Thus differences in nine-month rectus nerve neurotisation were dependent on differences in axon count.

Mean axon count was 754.0 (SD=393.34) in $R_9^{MMSB2}$, 1389.4 (SD=290.44) in $R_9^{MMS2}$, 1335.0 (SD=690.91) in $R_9^{VRBSB2}$ and 3357.8 (SD=1280.90) in $R_9^{VRBS2}$. Axon count of $R_9^{VRBS2}$ significantly exceeded that of $R_9^{MMS2}$ biopsies (p=0.010, t-test), whereas axon counts in $R_9^{MMSB2}$ and $R_9^{VRBSB2}$ biopsies were similar (p=0.141, t-test). Comparing axon counts of $R_9^{VRBS2}$ with $R_9^{VRBSB2}$ nerve biopsies and $R_9^{MMS2}$ with $R_9^{MMSB2}$ nerve biopsies revealed significantly fewer axons when the SB nerve graft was used in each case (p=0.014 and p=0.020 respectively, t-test).

These data were confirmed by comparison of total axon area. Mean total axon area was 4529.31 $\mu m^2$ (SD=1594.96) in $R_9^{MMSB2}$, 7643.37 $\mu m^2$ (SD=2145.59) in $R_9^{MMS2}$, 7234.63 $\mu m^2$ (SD=2279.28) in $R_9^{VRBSB2}$ and 15817.73 $\mu m^2$ (SD=4191.34) in $R_9^{VRBS2}$. Whilst

$R_9^{MMSB2}$ and $R_9^{VRBSB2}$ biopsies exhibited similar total axon areas (p=0.061, t-test), that of $R_9^{VRBS2}$ significantly exceeded that of $R_9^{MMS2}$ (p=0.005, t-test). In each case total axon area was significantly lower when the lower-capacity (SB) nerve graft was used, comparing $R_9^{VRBS2}$ with $R_9^{VRBSB2}$ and $R_9^{MMS2}$ with $R_9^{MMSB2}$ nerve biopsies (p=0.004 and p=0.031 respectively, t-test).

## 4.8. One- versus Two-Stage Surgery

Comparison of nerve morphometry data for one- and two-stage groups is summarised in Figure 4.5. As discussed earlier, a variable axon area/number relationship within nerve grafts may have resulted from variability in the exact biopsy position along the nerve graft length. For this reason, comparison of one- versus two-stage nerve graft neurotisation was made after expressing the latter as total axon area. There was no significant difference in mean total axon area comparing $SB_9^{MM1}$ with $SB_9^{MM2}$ (6401.12 $\mu m^2$, SD=2746.36 vs. 3647.46 $\mu m^2$, SD=1879.21 respectively, p=0.101), $SB_9^{VRB1}$ with $SB_9^{VRB2}$ (8099.70 $\mu m^2$, SD=3331.67, vs. 4094.67 $\mu m^2$, SD=4302.48 respectively, p=0.138) or $S_9^{VRB1}$ with $S_9^{VRB2}$ (25954.95 $\mu m^2$, SD=6106.44 vs. 27123.12 $\mu m^2$ respectively, SD=18216.41 respectively, p=0.895, all t-test). However $S_9^{MM1}$ nerve grafts exhibited significantly higher total axon areas than with $S_9^{MM2}$ grafts (15351.73 $\mu m^2$, SD=1879.43, vs. 5493.68 $\mu m^2$, SD=2199.01, p<0.001, t-test).

At the level of the rectus nerve, there was no significant difference between any corresponding one- and two-stage surgery groups in neurotisation expressed as axon

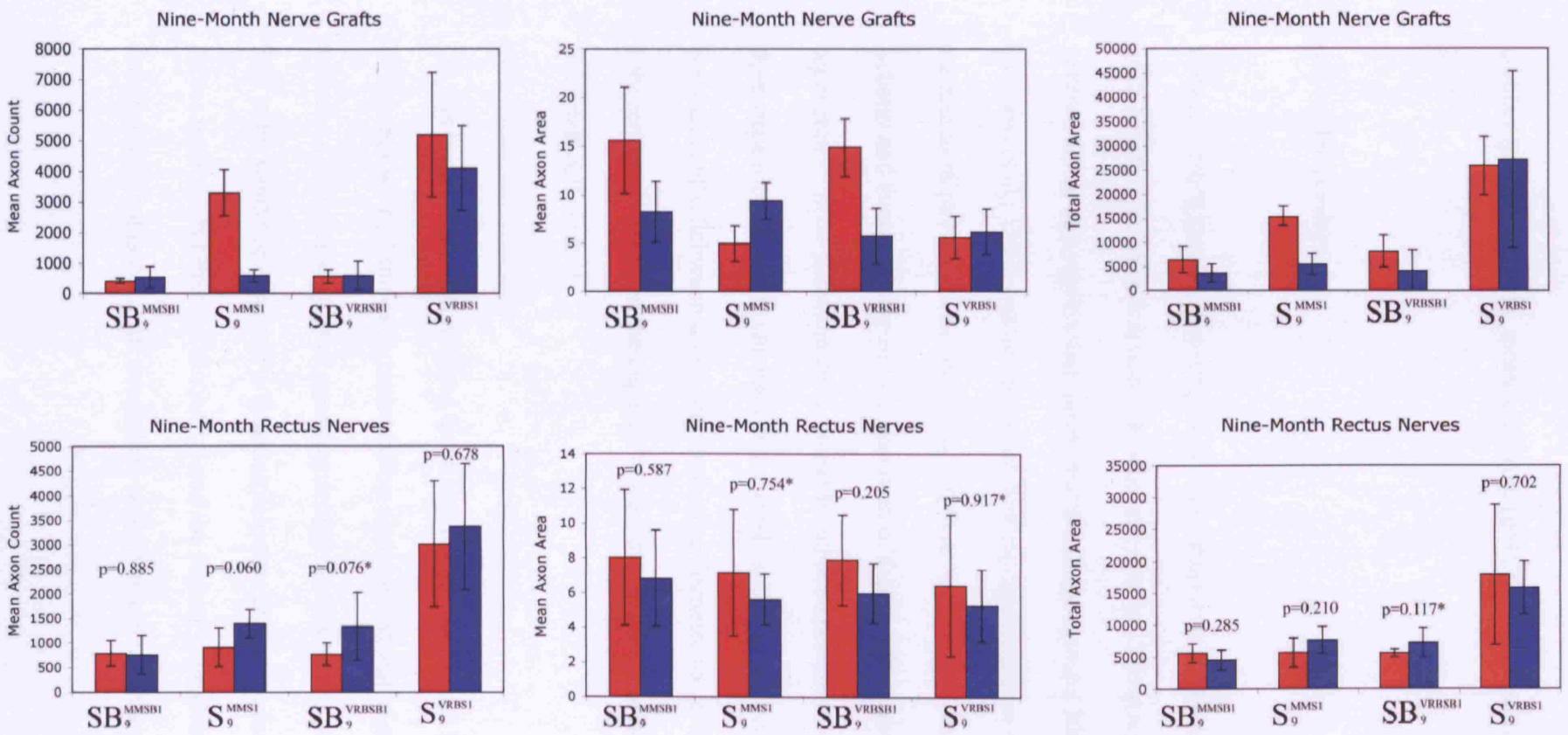



**Figure 4.5.** Nerve morphometry data for one- (red) and two-stage (blue) surgery groups (mean ± SD). Statistical tests performed using independent samples t-test unless data failed Levene's test for equality of variance, in which the Mann-Whitney rank sum test was used (*).

number (p=0.060-0.885), mean axon area (p=0.205-0.917) or total axon area (p=117-0.702).

## 4.9. Discussion

Characteristics known or suspected to affect axon transmission by a nerve graft include graft length, type, diameter and Schwann cell content. When a nerve graft is harvested, severed axons and myelin degenerate and are phagocytosed, but Schwann cells survive (Aguayo et al., 1977) and aid the passage of regenerating axons both through the formation of physical conduits (Guth, 1956) or 'Schwann tubes' (Letourneau, 1975; Scherer and Easter, 1984) and the secretion of factors which promote growth of axon regenerates. Since Schwann cells appear to enhance peripheral nerve regeneration in a dose-dependent manner (Guenard et al., 1992) and a correlation between the number of Schwann cells, Schwann tubes and total nerve diameter are probable, the relationship between axon width and the capacity to transmit axons could be partly mediated by Schwann cell content.

On this basis it was hypothesised that a low calibre nerve graft possessing fewer Schwann cells/tubes would limit axon transmission across it and that of any coapted end organ, whereas a high-capacity graft encourages high fidelity transmission of axons to muscle. The latter would be expected to produce disparate end organ function from variable motor input (as is observed clinically) and the former to produce motor nerve independent, but nerve graft dependent, end organ reinnervation.

This hypothesis predicts that the use of a low diameter nerve graft containing few myelinated fibres at the time of harvest, and therefore generating a Schwann tube-poor nerve graft, could standardise axon transmission between donor nerve and FMT notwithstanding variable donor nerve input, whereas no such standardisation would occur when using a larger diameter interposed nerve graft originally containing many myelinated fibres. This involves an assumption that the number of myelinated fibres in a nerve correlates with the number of vacated Schwann tubes when it is harvested for use as a nerve graft. Since Schwann tubes are derived from the spaces enclosed by the basement membranes surrounding the Schwann cells of peripheral myelinated nerve fibres, this assumption seems reasonable.

Nerve morphometry was performed with two aims: firstly, at 0 months, to validate the selection of motor nerves and nerve grafts described in Chapter 2; and secondly, at 3 and 9 months, to test the above prediction.

### 4.9.1. Selection of Motor Nerves and Nerve Grafts

Motor nerve selection for the current study was guided by previous experimental work, both from this unit and elsewhere. Nehrer-Tairych (Nehrer-Tairych et al., 2000) previously evaluated the buccal branch of the facial nerve using semi-automated (digitizer pen) morphometry with sampling to yield a mean nerve fibre count of 3209 and mean fibre diameter of 7.45μm, equating to a mean area of 43.6μm$^2$ (assuming circular

nerve fibres). MacQuillan (MacQuillan, 2005) utilised the marginal mandibular (MM) and ventral ramus of buccal (VRB) facial nerve branches to compare different reinnervating axon loads on rectus femoris FMT reinnervation and force production in the New Zealand white rabbit model. Also using semi-automated (digitizer pen) morphometry without sampling (see Chapter 3 for discussion of morphometric methods), mean axon counts of 635 or 731 in the MM and of 2627 or 3912 in the VRB (6 and 9 month study groups respectively) were identified. Mean axon areas were 12μm² or 15μm² in the MM and 45μm² and 40μm² in the VRB (6 and 9 month study groups respectively). Although Nehrer-Tairych measured nerve fibre area (area of axon and myelin) whilst MacQuillan assessed axon area (excluding myelin thickness), results were broadly concordant.

These studies indicated that the MM and VRB might provide suitable donor nerves for the present study. This was confirmed by histomorphometric examination of six biopsies of each nerve at the time of first stage surgery., showing that $MM_0$ nerves contained significantly fewer similarly-sized myelinated axons compared with $VRB_0$. Interestingly, whilst $MM_0$ and $VRB_0$ mean axon counts and $MM_0$ mean axon area agreed with those of previous studies (505, 3853 and 18μm$^2$ respectively), measured mean axon area for $VRB_0$ (19μm$^2$) was considerably lower than previously (Nehrer-Tairych et al., 2000; MacQuillan, 2005). In the study by MacQuillan, morphometric quantification was performed at different time points (6 and 9 months following FMT, rather than before FMT) and results may not be directly comparable with those of the current study. In the study by Nehrer-Tairych, the difference may be accounted for by the need to estimate

area from diameter in the latter and the measurement of fibre rather than axon diameter. Additionally, the method used for the current study may have had greater accuracy in detecting small axons than previous methods (Urso-Baiarda and Grobbelaar, 2006), producing a slightly lower mean axon area and higher axon count than recorded elsewhere.

Nerve graft characteristics considered advantageous for this study were: two nerves of different axon area and/or number which could be harvested to the same length; sensory rather than motor nerves, to resemble the clinical situation of interpositional nerve grafting for FMT reinnervation; graft harvest through the FMT operative incision, to minimise animal morbidity; and a non-crippling donor defect, since final experimental procedures took place 9 months after the first.

Several previous studies have used autologous nerve graft for muscle reinnervation in a rabbit model, including sural, posterior tibial (Matsuzaki et al. 2005), sciatic and saphenous nerve grafts (Koller et al., 1997). Of these, only the sural and saphenous were purely sensory and only the saphenous could be harvested via a thigh incision. There was no precedent in the literature for a second nerve graft of different neural content and similar length suitable for comparison with the saphenous in this study. Through dissection, however, it was discovered that a 3cm length of the much narrower medial branch of the saphenous nerve (SB) could also be obtained via a thigh incision. Six biopsies of each of the saphenous and medial saphenous nerves were compared histomorphometrically to confirm their suitability for use in this study, showing that $SB_0$ contained both significantly fewer (1045 vs 2553) and smaller ($6\mu m^2$ vs. $8\mu m^2$)

myelinated axons and had a lower overall fascicular cross sectional area compared with $S_0$. These observations supported the use of S versus SB as nerve grafts in this study.

### 4.9.2. Three-Month Nerve Morphometry

Nerve grafts belonging to animals from all four two-stage groups were examined and quantified at 3 months. All exhibited statistically similar mean axon areas (2.8-3.8$\mu m^2$) that were, in all cases, greatly reduced compared with both original motor nerve and original nerve graft axon areas. Reduced diameter of axon regenerates within a nerve graft has been shown previously (Ansselin and Davey, 1988) and is consistent with the formation of slender axon sprouts as an early event in nerve regeneration (Morris et al., 1972; Kim et al., 1990).

Since mean three-month nerve graft axon area was similar across all groups, axon number was the major determinant of the extent of graft neurotisation. Axon count was significantly higher in $S_3^{VRB}$ compared with $S_3^{MM}$ nerve grafts (2453 vs. 1634 respectively), but similar in $SB_3^{VRB}$ and $SB_3^{MM}$ grafts (1035 vs. 906). Whilst use of the SB nerve graft significantly reduced three-month graft neurotisation for each motor nerve (i.e. $SB_3^{VRB}$ vs. $S_3^{VRB}$, and $SB_3^{VRB}$ vs. $SB_3^{VRB}$). These observations were consistent with the hypothesis that the SB nerve graft could both limit and reduce variability of downstream neurotisation despite variable motor input.

Previous research has shown that a divided peripheral nerve sends multiple axon sprouts from each nerve fibre (Morris et al., 1972; Kim et al., 1990) and consequently the total number of myelinated fibres distal to a nerve transection initially increases (Jenq and Coggeshall, 1985). This is in keeping with some but not all of the three-month nerve graft data from this study: the proportion of axons crossing the proximal coaptation into the nerve graft from MM was 179.41% for $SB_3^{MM}$ and 323.60% for $S_3^{MM}$, consistent with axon sprouting (along with the reduction in mean axon area noted earlier). Previous research suggests that motor unit expansion for the facial nerve occurs maximally by a factor of 2-3 (Frey, 1998)[7], so 320% axon sprouting occurring in $S_3^{MM}$ may represent the highest possible axon count that could physiologically be achieved within the nerve graft.

Conversely, no increase in nerve graft axon count was seen when the larger VRB donor nerve was used. At three months, the percentage of VRB axons crossing the proximal coaptation into the nerve graft was 26.86% in $SB_3^{VRB}$ and 63.65% in $S_3^{VRB}$. Thus it appears either that transected rabbit VRB cannot elicit sufficient axon sprouting to match its original axon count, or that neither the SB nor the S nerve grafts were capable of transmitting most of the sprouts produced. In support of the latter over the former, earlier studies using reversed sections of VRB as autologous nerve grafts for repair of segmental VRB defects found similar axon counts in both VRB and nerve graft (3876 ± 1512 vs. 3995 ± 1209 myelinated axons (Spector, 1997). Whilst earlier studies therefore have

[7] compared with at least a fivefold increase by peripheral nerves
Brown MC, Jansen JK, Van Essen D (1976) Polyneuronal innervation of skeletal muscle in new-born rats and its elimination during maturation. J Physiol 261:387-422,
Thompson W, Jansen JK (1977) The extent of sprouting of remaining motor units in partly denervated immature and adult rat soleus muscle. Neuroscience 2:523-535,
Gorio A, Marini P, Zanoni R (1983) Muscle reinnervation--III. Motoneuron sprouting capacity, enhancement by exogenous gangliosides. Neuroscience 8:417-429.

demonstrated increased or maintained axon numbers across a coaptation, the current data suggests that this is conditional upon the ability of the distal segment, in this case the nerve graft, to transmit those sprouts. This is further supportive evidence of the potential use of a nerve graft in limiting axon transmission between motor nerve and target organ.

### 4.9.3. Nine-Month Nerve Morphometry

Whilst nine-month nerve morphometry data is complicated by the need for several group comparisons occurring in different nerves (graft or rectus) and repeated for one- and two-stage surgery, it is possible to draw several generalisations from the data that help to clarify the overall picture.

There was unexpected variability in the relationship between axon number and axon area between equivalent one- and two-stage nerve graft biopsies. For instance, $S_9^{MM1}$ nerve grafts contained many axons (3300) of low area ($5\mu m^2$), compared with $S_9^{MM2}$ nerve grafts (580 axons of mean area $9\mu m^2$). Other, albeit less dramatic, differences in the axon count/mean area relationship between corresponding one- and two-stage nerve graft biopsies were seen, with no clear pattern consistently favouring one- or two-stage surgery. A simple potential explanation was that, due to progressive axon ramification along a nerve graft length, biopsies taken increasingly distally would be characterised by increasingly small but numerous axons. Although efforts were made to take nerve graft biopsies from equivalent positions this was often made difficult by the tendency of redundant nerve graft length to form shortened coiled bundles as they became encased in

scar tissue between operative stages, and it is possible that non-equivalent nerve graft biopsy sampling contributed to the observed variation in axon number and mean area.

In order to obviate such an effect, it was necessary to compare nine-month nerve graft neurotisation expressed as total axon area. For both one- and two-stage surgery groups, total axon area within the nerve graft at 9 months correlated with the size of motor nerve input when a large interposed nerve graft was used (26000$\mu m^2$ vs. 15400$\mu m^2$ for $S_9^{VRB1}$ and $S_9^{MM1}$ respectively) but not when a small interposed nerve graft was used (8100$\mu m^2$ vs. 6400$\mu m^2$ for $SB_9^{VRB1}$ and $SB_9^{MM1}$ respectively). This again supports the hypothesis that it may be possible to standardise graft neurotisation, and potentially also more distal neurotisation, by making use of properties of the interposed nerve graft itself.

Mean axon area for rectus nerves from all four one-stage and all four two-stage study groups were similar (6-8$\mu m^2$ and 5-7$\mu m^2$ respectively), and thus the main determinant of neurotisation of the FMT neural pedicle was axon number, although for confirmation neurotisation was also expressed and compared as total axon area. For both one- and two-stage surgery groups, greatest axon counts and total axon areas were seen in FMT neural pedicles reinnervated using a combination of the largest motor nerve (VRB) and the largest interposed nerve graft (S). Consistent with the study hypothesis, in all cases, the magnitude of motor nerve input correlated with the amount of neural pedicle neurotisation[8] when the S nerve graft was used but not when the SB nerve graft was used. This is in keeping with nine-month nerve graft data and supports the hypothesis that use

[8] Neurotisation being expressed either as axon count or as total axon area.

of the interposed nerve graft may also enable regulation of reinnervation of structures distal to the nerve graft itself, potentially permitting standardisation of clinical outcome.

Comparisons were also made for either the S or SB nerve graft combined with a constant motor nerve, in order to determine whether use of the latter impaired neurotisation by both MM and VRB to a similar extent, at the level of both the nerve graft and the rectus nerve. Such comparison revealed that the SB nerve graft consistently (i.e. for both one- and two-stage surgery groups, at both nerve graft and rectus nerve levels) impaired neurotisation by the VRB motor nerve, but that this impairment was less consistent for the MM motor nerve. For instance, neurotisation of $S_9^{MM1}$ significantly exceeded that of $SB_9^{MM1}$, but neurotisation of $S_9^{MM2}$ and of $SB_9^{MM1}$ were similar. Similarly, $R_9^{MMS2}$ neurotisation[2] significantly exceeded that of $R_9^{MMSB2}$ whereas $R_9^{MMSB1}$ and $R_9^{MMS1}$ neurotisation were statistically similar. This inconsistency could result from the fact that the neural content of VRB greatly exceeded that of MM, so the limiting influence of SB on axon transmission was much larger for the former than the latter. Thus, the large difference in neurotisation between $R_9^{VRBS1/2}$ and $R_9^{VRBSB1/2}$ was consistently detectable, whereas a smaller difference in neurotisation between $R_9^{MMS}$ and $R_9^{MMSB}$ sometimes achieved statistical significance (in the case of two-stage groups) and at other times did not (in the case of one-stage groups).

#### 4.9.4. One- vs. Two-Stage Surgery

Previous research comparing axon regeneration across a sheep saphenous nerve graft either with or without distal target muscle connection found inconsistent results, with 'increases or decreases [in the regenerated fibre population] without **any** correlation to the interval without target muscle' (Frey et al., 1996). In the present study, reinnervation was similar between one- and two-stage nerve biopsies at the level of the nerve graft at nine months for three of the four study groups, there being statistically superior reinnervation (in terms of total axon area) in one- compared with two-stage $S_9^{MM}$ biopsies. Whilst this was in keeping with superior reinnervation seen previously amongst one-stage FMTs (Mackinnon et al., 1988; Rab et al., 2006) it is difficult to submit a physiological explanation for the expression of this in $S_9^{MM1}$ but not in the remaining groups, and it is possible that this result was a chance effect. At the level of the rectus femoris, reinnervation was identical comparing all respective one- and two-stage surgery groups.

To summarise, nerve morphometry data showed that:

the SB nerve contained fewer and smaller myelinated axons than S. The study assumes that this will result in fewer Schwann tubes in the former than the latter when used as a nerve graft;

the neural content of the VRB branch of the facial nerve exceeded that of MM, confirming previous research;

when the SB interposed nerve graft was used, neurotisation of both the graft itself (at both 3 and 9 months) and the more distal rectus nerve (at 9 months) was similar irrespective of the motor nerve used, despite the difference in neural content of the two motor nerves. Conversely, when S was the interposed nerve graft, nerve graft and rectus nerve neurotisation was related to the magnitude of motor nerve neural content;

that the SB interposed nerve graft consistently (at the level of the graft and rectus nerve, for both one- and two-stage surgery) impaired neurotisation by a VRB motor nerve compared with the use of an S interposed nerve graft; whereas its effect on MM neurotisation was less consistent, sometimes impairing downstream neurotisation and at other times having no significant effect. In explanation, the SB nerve graft may have been almost completely able to accommodate MM axon sprouts, so its effect in limiting MM neurotisation was marginal and variably significant.

# CHAPTER 5

# RESULTS:

# MUSCLE PHYSIOLOGY

## 5.1. Introduction

Extensive previous research has assessed functional recovery of microneurovascular functional muscle transfers (FMTs). The earliest work compared orthotopic FMTs (back into the original tissue bed) with heterotopic transfers (to another location) using the dog gracilis model (Tamai et al., 1970). The study found favourable histological results and near-normal EMG studies 5 months post-transfer in all FMTs regardless of orthotopic or heterotopic transfer, concluding that "perfect recovery of function" had been achieved although functional recovery in terms of force production had not been determined.

That study formed the basis for clinical FMTs, with encouraging early results (Shanghai Sixth People's Hospital 1976, (Harii et al., 1976), and further experimental work. The latter included a systematic examination of the individual muscle insults occurring during FMT using the rabbit rectus femoris model (Terzis et al., 1978), demonstrating a 20% tetanic force deficit following microneurovascular repair alone, a 50% deficit following tenotomy alone, and 75% force reduction following orthotopic or heterotopic muscle transfer. Thus functional recovery following full FMT was unexpectedly poor compared with clinical experiences achieved at the time, and called into question the usefulness of FMT as a technique.

The inconsistency between clinical and experimental observations spurred others to investigate functional recovery of FMTs further. Using the same rabbit model, approximately 70% weight-adjusted force production was achieved 6 months after

heterotopic transplantation (Frey 1983), and 32% of control tetanic force was generated by FMTs 90 days after orthotopic transfer (Guelinckx et al., 1984). These represented greater force production by FMTs after shorter recovery periods than in the earlier work (Terzis et al., 1978) and provided renewed encouragement to those interested in the reconstructive potential of FMTs. Whilst full recovery of function (compared with unoperated contralateral muscle) appeared unattainable, FMTs could nonetheless achieve significant useful contractile function (Frey et al., 1983b).

In the clinical situation, FMTs are always transferred heterotopically (to a non-equivalent anatomical site). Therefore the effects of both different reinnervating motor inputs on identical transferred FMTs, and of different FMTs on constant reinnervating motor inputs, are relevant experimental questions. The latter was addressed by a series of experiments in which a constant nerve supply was used to reinnervate muscle of increasing mass (Frey, 1998). Replacing the rabbit scutuloauricularis ear erector muscle with a double-sized muscle (either a portion of rectus femoris or the entire pectoralis descendens) elicited 185-188% tetanic force of contralateral control scutuloauricularis, whereas orthotopically-transplanted scutuloauricularis generated 88% tetanic force. Further increases in FMT mass further did not further increase force generation, however, with triple-sized muscle producing only 173% tetanic force of control (Giovanoli et al., 2003). The authors concluded that further increases in force production were not possible by increasing FMT mass once the upper limit of motor unit expansion by reinnervating axons had been achieved.

Most clinical facial reanimation techniques use similarly sized muscle transfers (generally either the entire pectoralis minor or portions of gracilis or latissimus dorsi, depending on individual surgeon's preference) but different sized motor inputs (dependent on variable facial nerve anatomy and random differences in nerve recovery). Thus the effect of reinnervating constant muscle mass with varying motor input is likely of greater clinical relevance. Previous research using a rabbit rectus femoris model of facial reanimation by FMT has shown that ultimate force production correlates with the magnitude of donor motor input (MacQuillan, 2005). This may contribute to the variable clinical outcome of FMT with cross-facial nerve grafting (CFNG) (see Chapter 1, Section 1.3), the corollary being that outcome could be regulated by precisely controlling FMT neurotisation.

The nerve graft represents a potential site of FMT force modulation in most clinical cases since FMT for unilateral facial palsy usually mandates a CFNG to overcome the length deficit between neural pedicle and contralateral facial nerve. However, no previous research has investigated a potential role of nerve grafts in controlling force production by a FMT. Since nerve grafts vary in the degree to which they are supportive of axon regeneration, based on factors including physical characteristics and Schwann cell content (Scherer and Easter, 1984) (termed high and low capacity nerve grafts), it may be possible to achieve nerve graft-dependent, and motor input-independent, FMT neurotisation by use of a low capacity nerve graft. This hypothesis is supported by nerve morphometry results discussed in Chapter 4. For this approach to represent a possible strategy for standardising clinical outcome, however, FMT reinnervation must correlate

with force production and accordingly with clinical outcome. The current experimental section aims to examine the presence of such a relationship.

## 5.2. Aims

To compare the effect of different sized motor nerve inputs combined with different diameter nerve grafts on physiological properties of a FMT.

## 5.3. Method

Nine months after either one-stage surgery or the first procedure for two-stage surgery groups (see Chapter 2 Section 2.4), FMTs were approached via the original facial scar, isolated on their neurovascular pedicles, and rigidly attached at their origin to a metal frame and at their insertion to a computer-linked force transducer (Chapter 2 Section 2.6). They were then electrically stimulated midway along the nerve graft by means of two platinum electrodes separated by 5mm. The tension placed across the muscle was adjusted until spike contraction force to 100Hz stimulation was maximal (Figure 5.1A), and then stimulated at this tension incrementally from 10-100Hz frequencies (Figure 5.1B). In this way it was possible to construct a force-frequency curve for each FMT. Immediately after this, both tetanic force production and fatigue rate were measured by continuous stimulation at 100Hz (Figure 5.1C). Fatigue rate was calculated from the slope of tetanic force decay against time. The process was repeated for control rectus

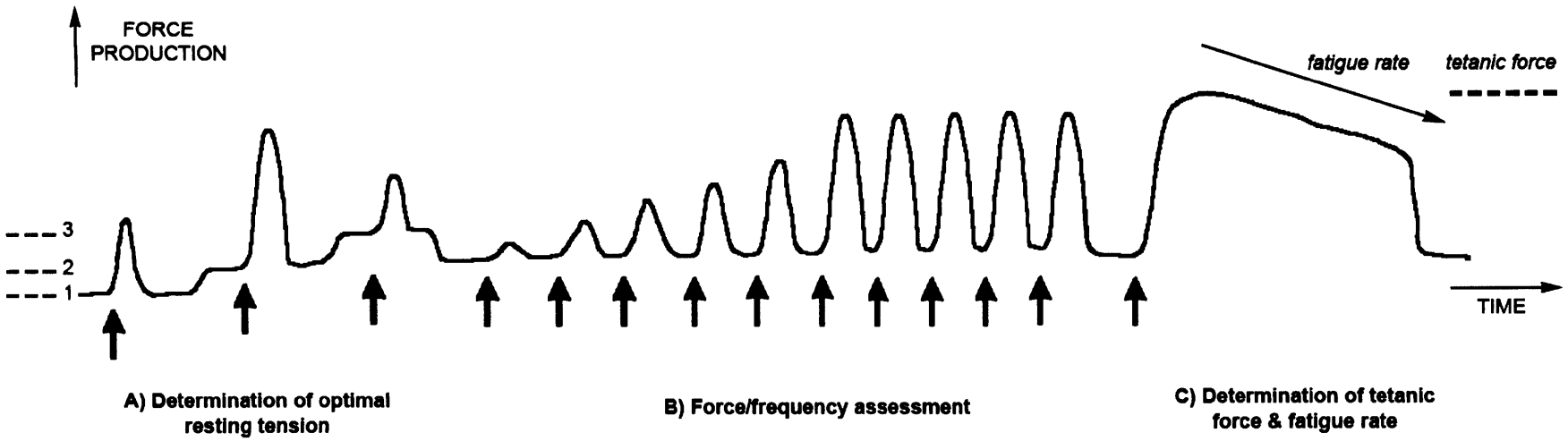



**Figure 5.1.** Illustration of typical stimulation force/time recording. Heavy arrows represent electrical stimulations. A) Optimal resting tension is determined as the resting tension producing maximal twitch tension to 100Hz stimulation. This resting tension (2) is used for all subsequent recordings. B) Force/frequency relationships are determined by measuring twitch response to incremental increases in stimulation frequency from 10-100Hz. C) Finally, sustained 100Hz stimulation is used to determine tetanic force and fatigue rate.

femoris muscles dissected from the right thigh. The method was identical except that the origin was left attached to the pelvis, since the pelvis could be fixed rigidly to the operating table for accurate measurement of force production.

Statistical comparison of force frequency curves for individual study groups across all stimulation frequencies was performed by means of 2-way ANOVA. Muscle fatigue rate between groups was compared using 1-way ANOVA followed by post-hoc Tukey analysis. Statistical tests were performed using Statistics Package for the Social Sciences (SPSS) version 11.0.4 for Mac OS X.

## 5.4. Terminology

The group terminology used in this chapter is identical to that described in Chapter 4 Section 4.4 (superscript). Thus MMS1 refers to the study group in which FMTs were reinnervated using the marginal mandibular (MM) facial nerve branch via an interposed saphenous nerve graft (S), all performed at a single operative stage (1). VRBSB2 refers to study groups in which FMTs were reinnervated by the ventral ramus of buccal branch (VRB) of the facial nerve via an interposed medial saphenous branch nerve graft (SB), performed over two-stages (2).

## 5.5. Absolute Force Production

### 5.5.1. One-Stage Surgery Groups

Mean absolute force production ($F_A$) generated by FMTs is shown in Figure 5.2. Greatest $F_A$ was generated by FMTs from the VRBS1 group (mean maximal tetanic force=191.52g, SD=122.93), followed in magnitude by MMS1 (mean maximal tetanic force=51.39g, SD=41.17), MMSB1 (mean maximal tetanic force=50.92g, SD=24.38) and VRBSB1 (mean maximal tetanic force=46.16g, SD=43.76) groups.

In order to assess the effect of motor nerve input magnitude on $F_A$ when using either S or SB grafts, a comparison of $F_A$ (across all stimulation frequencies) was made between MMS1 and VRBS1 and between VRBSB1 and MMSB1. This showed that $F_A$ of VRBS1 FMTs significantly exceeded that of MMS1 FMTs ($F=54.564$, $p<0.001$) whereas $F_A$ of MMSB1 and VRBSB1 FMTs were statistically similar ($F=0.022$, $p=0.882$). Thus $F_A$ was independent of the magnitude of motor input when a small (SB) but not when a large (S) nerve graft was used.

To assess whether the use of a smaller SB nerve graft reduced $F_A$ of both large (VRB) and small (MM) motor inputs, $F_A$ of MMSB1 was compared with that of MMS1, and $F_A$ of VRBSB1 was compared with that of VRBS1. $F_A$ of VRBSB1 was significantly less than that of VRBS1 FMTs ($F=50.905$, $p<0.001$), but $F_A$ of MMSB1 and MMS1 were

similar (F=0.123, p=0.727), indicating that the low capacity interposed nerve graft reduced absolute force production of FMTs reinnervated with the larger motor input only.

### 5.5.2. Two-Stage Surgery Groups

Mean $F_A$ generated by two-stage FMTs is shown in Figure 5.3. A similar overall pattern was seen amongst two-stage surgery groups as in one-stage surgery groups: greatest maximum tetanic force was produced by VRBS2 group FMTs (mean maximal tetanic force=86.70g, SD=42.10), followed by those from the MMS2 (mean maximal tetanic force=64.11g, SD=12.38), MMSB2 (mean maximal tetanic force=58.24g, SD=7.80) and VRBSB2 (mean maximal tetanic force=44.85g, SD=20.84) groups.

$F_A$ between VRBS2 and MMS2 and between VRBSB2 and MMSB2 was compared to assess the effect of motor nerve input magnitude when using either S or SB grafts. This showed that $F_A$ by VRBS2 FMTs was significantly greater than that of MMS2 FMTs (F=5.390, p=0.023). Surprisingly, however, $F_A$ by MMSB2 was slightly but significantly higher than that of VRBSB2 FMTs (F=6.810, p=0.011).

$F_A$ was compared across all stimulation frequencies between MMSB2 and MMS2 FMTs, and between VRBSB2 and VRBS2 FMTs, to assess whether the use of the lower capacity SB nerve graft reduced $F_A$ for both large (VRB) and small (MM) motor inputs. $F_A$ by VRBSB2 FMTs was significantly lower than that of VRBS2 FMTs (F=31.346, p<0.001), and $F_A$ by MMSB2 was significantly less than that of MMS2 (F=14.978, p<0.001),

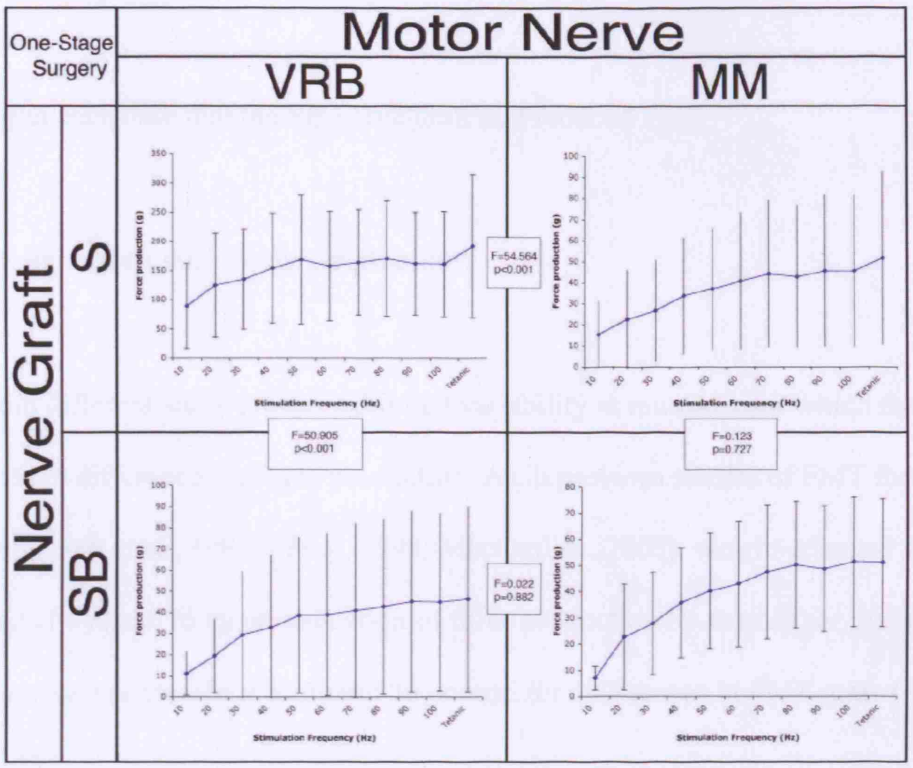



**Figure 5.2.** Mean absolute force/stimulation frequency relationships for one-stage FMTs. Points represent mean ± SD. Statistical comparisons made using 2-way ANOVA.

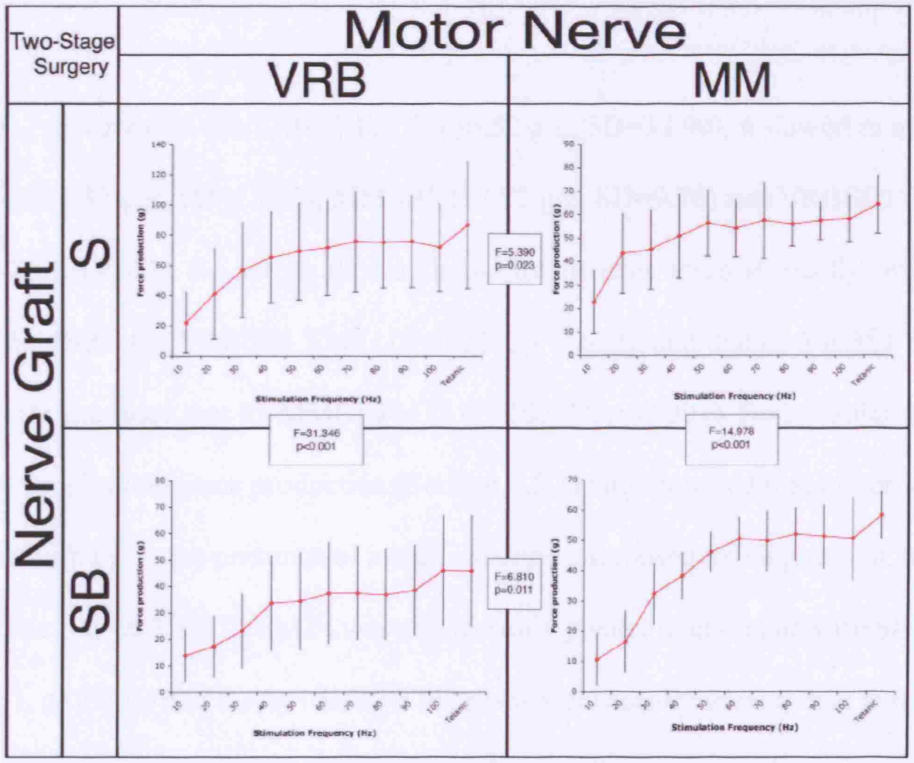



**Figure 5.3.** Mean absolute force/stimulation frequency relationships for two-stage FMTs. Points represent mean ± SD. Statistical comparisons made using 2-way ANOVA.

indicating in each case that the SB nerve graft had reduced $F_A$.

## 5.6. Weight-Adjusted Force Production

FMTs from different study groups exhibited variability in muscle mass which may have contributed to differences in force production. As in previous studies of FMT force production (Frey et al., 1983b; Frey, 1998; MacQuillan, 2005), weight-adjusted force production ($F_W$) (providing an indication of force production by muscle per gram of whole muscle) was therefore compared to control for differences in FMT mass (Figures 5.4 and 5.5).

### 5.6.1. One-Stage Groups

Tetanic $F_W$ was greatest for VRBS1 FMTs (46.52 g/g, SD=32.90), followed in magnitude by MMS1 (19.37 g/g, SD=15.83), MMSB1 (17.73 g/g, SD=9.76) and VRBSB1 (12.70 g/g, SD=11.12) FMTs. $F_W$ across all stimulation frequencies was statistically similar between MMSB1 and VRBSB1 FMTs ($F=0.231$, $p=0.632$), and that by VRBS1 FMTs significantly exceeded that by MMS1 FMTs ($F=16.994$, $p<0.001$). Both results were in keeping with absolute force production (Section 5.5.1) and indicated that motor input correlated with $F_W$ in the presence of a high capacity interposed nerve graft but not a low capacity one. $F_W$ by VRBS1 FMTs was significantly greater than that of VRBSB1 FMTs ($F=40.067$, $p<0.001$) and $F_W$ by MMS1 FMTs was significantly greater than that of

MMSB1 FMTs (F=10.932, p=0.001), indicative that use of the low capacity interposed nerve graft reduced $F_W$ of FMTs reinnervated by either MM or VRB motor nerves.

### 5.6.2. Two-Stage Groups

Tetanic $F_W$ was greatest for VRBS2 FMTs (21.69 g/g, SD=4.80), followed in magnitude by VRBSB2 (19.11 g/g, SD=12.01), MMSB2 (16.86 g/g, SD=5.74) and MMS2 (14.14 g/g, SD=3.47) FMTs. $F_W$ by MMSB2 and VRBSB2 FMTs were statistically similar (F=3.702, p=0.058), whereas $F_W$ by VRBS2 FMTs was significantly greater than that of MMS2 FMTs (F=37.998, p<0.001). Thus, as for one-stage groups, $F_W$ correlated with motor input magnitude only when a high capacity interposed nerve graft was used. $F_W$ by VRBS2 FMTs was significantly greater than that of VRBSB2 FMTs (F=64.870, p<0.001), whereas $F_W$ by MMS2 FMTs was statistically similar to that of MMSB2 FMTs (F=0.767, p=0.384). Therefore, in the two stage surgery groups, the low capacity interposed nerve graft reduced $F_W$ by FMTs reinnervated by the larger motor nerve only.

## 5.7. Control-Adjusted Weight-Adjusted Force Production

Group comparisons made for FMT $F_W$ (Section 5.6) were repeated for corresponding control rectus femoris muscles in order to check for significant between-group differences in $F_W$ of unoperated muscle. Some significant differences were observed in

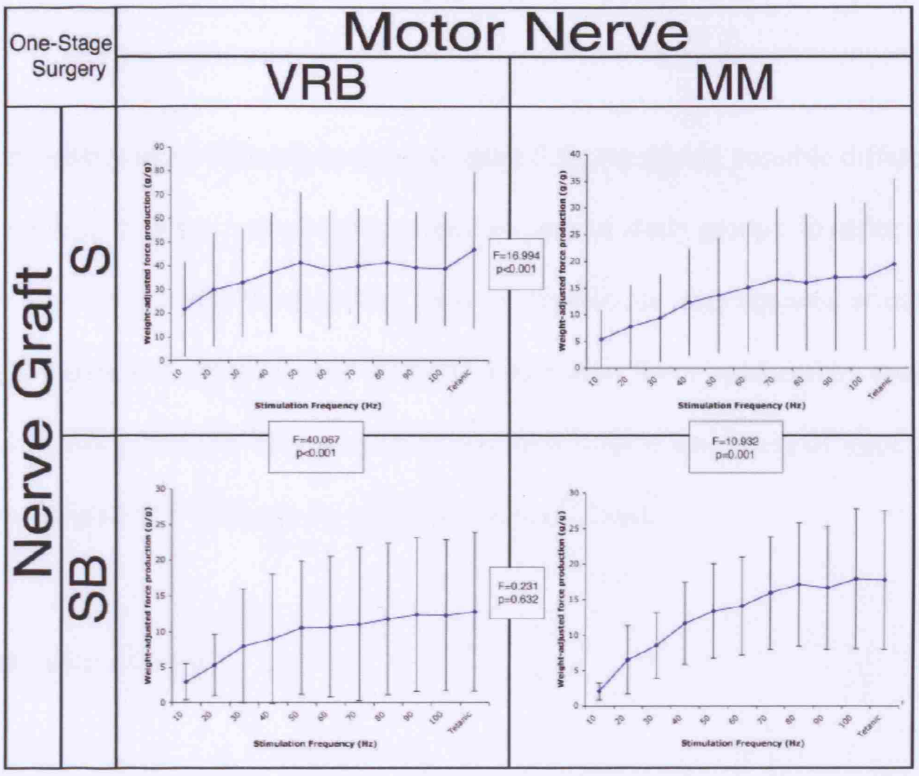



**Figure 5.4.** Mean weight-adjusted force/stimulation frequency relationships for one-stage groups. Points represent means ± SD. Statistical comparisons made using 2-way ANOVA.

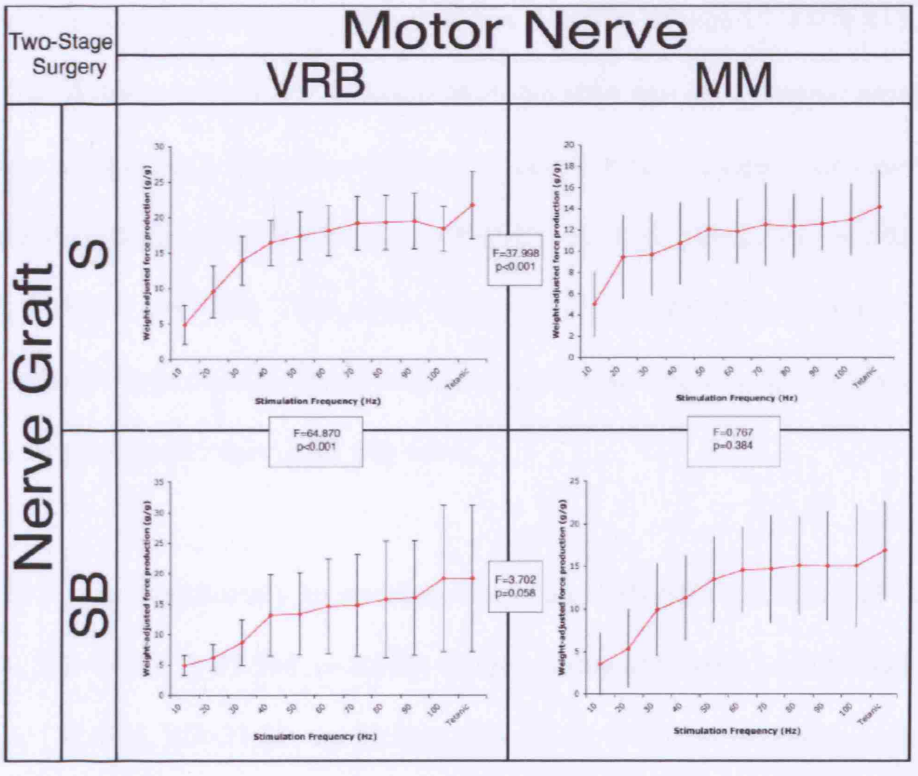



**Figure 5.5.** Mean weight-adjusted force/stimulation frequency relationships for two-stage groups FMTs. Statistical comparisons made using 2-way ANOVA.

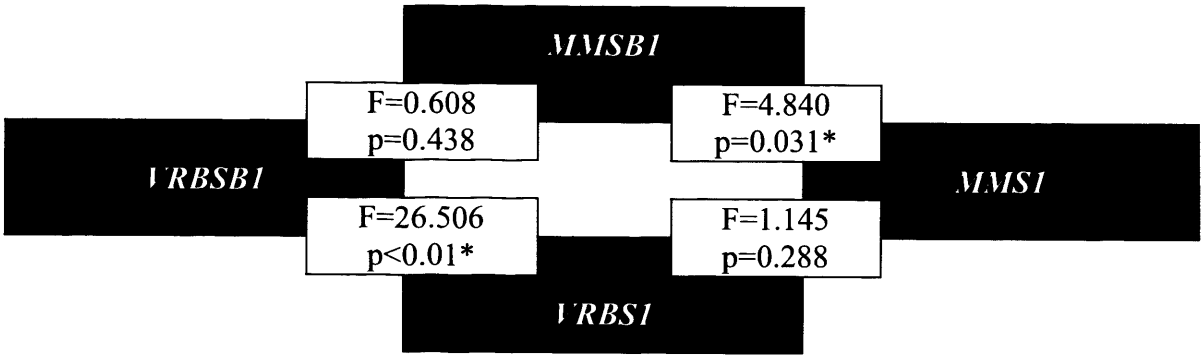
$F_W$ between control rectus femoris muscles (Figure 5.6), signifying possible differences in overall strength between animals randomised to various study groups. In order to control for this this potential confounding factor, comparisons were repeated after re-expressing $F_W$ data as a percentage of the maximum tetanic force produced by control rectus femoris muscle ($F_{CW}$). This has previously been used as a measure of force in studies comparing FMT force production (MacQuillan 2006).

### 5.7.1. One-Stage Groups

Control-adjusted weight-adjusted force production ($F_{CW}$) by one-stage surgery FMTs is shown in Figure 5.7. In keeping with $F_A$ and $F_W$ data, $F_{CW}$ of VRBS1 was significantly greater than that of MMS1 (64.74%, SD=28.81, vs. 32.98%, SD=26.19, F=39.433, $p<0.001$). The difference in force production between large and small reinnervating motor nerves was greatly reduced, and in fact reversed to a small extent, when the low capacity nerve graft was used (MMSB1 vs. VRBSB1: 32.00%, SD=21.23, vs. 20.33%, SD=14.55; F=5.669, p=0.020). Thus, as for $F_A$ and $F_W$, $F_{CW}$ correlated with motor input magnitude when a large capacity interposed nerve graft was used but did not when a small capacity interposed nerve graft was used.

$F_{CW}$ of VRBS1 was significantly greater than that of and VRBSB1 (64.74%, SD=28.81, vs. 20.33%, SD=14.55, F=83.564, $p<0.001$) whereas those of MMSB1 and MMS1 FMTs were similar (32.00%, SD=21.23, vs. 32.98%, SD=26.19, F=0.030, p=0.864), indicating

**One-Stage Groups**




**Two-Stage Groups**

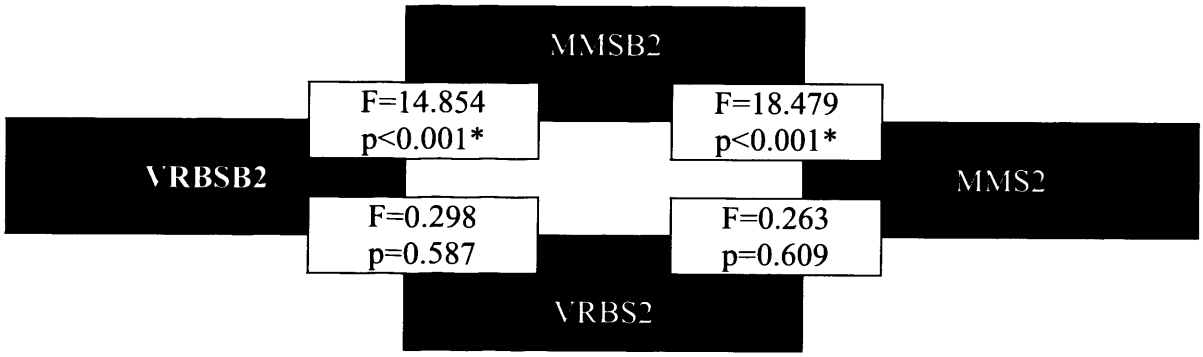



**Figure 5.6.** Summary of statistical comparisons of weight-adjusted force between contralateral non-transferred rectus femoris muscles. Significant differences are denoted by an asterisk.

that the low capacity interposed nerve graft reduced $F_{CW}$ by FMTs reinnervated by VRB but not by those reinnervated by MM.

### 5.7.2. Two-stage Groups

$F_{CW}$ by two-stage surgery FMTs is shown in Figure 5.8. Consistent with $F_A$ and $F_W$ data, $F_{CW}$ by VRBS2 FMTs was significantly greater than that of MMS2 (53.06%, SD=15.19, vs. 27.67%, SD=8.52, F=56.398, $p<0.001$). $F_{CW}$ of VRBSB2 FMTs also exceeded that of MMSB2 FMTs, although the difference was greatly attenuated (32.21%, SD=17.19, vs. 23.87%, SD=6.46, F=5.645, p=0.020). $F_{CW}$ of both VRBS2 and MMS2 FMTs were significantly greater than those of respective VRBSB2 and MMSB2 FMTs (VRBS2 vs. VRBSB2: 53.06%, SD=15.19, vs. 32.21%, SD=17.19; F=48.157, $p<0.001$; MMS2 vs. MMSB2: 27.67%, SD=8.52, vs. 23.87%, SD=6.46; F=8.976, p=0.004).

## 5.8. Muscle Fatigue

Fatigue rate for control rectus femoris muscles and for FMTs from each study group are shown in Figure 5.9. Unoperated rectus femoris muscles exhibited much greater fatigue rates than FMTs (Fatigue rates: Mean FMT=1.815g/s, SEM 0.270; mean control rectus=24.730g/s, SEM 1.502). Within control and FMT study groups, there was no overall difference between individual subgroups (FMTs: F=1.993, p=0.087; control muscles: F=0.694, p=0.677). Tukey analysis further showed that there was no significant difference between fatigue rate for any two FMT or control muscle groups (p=0.300-

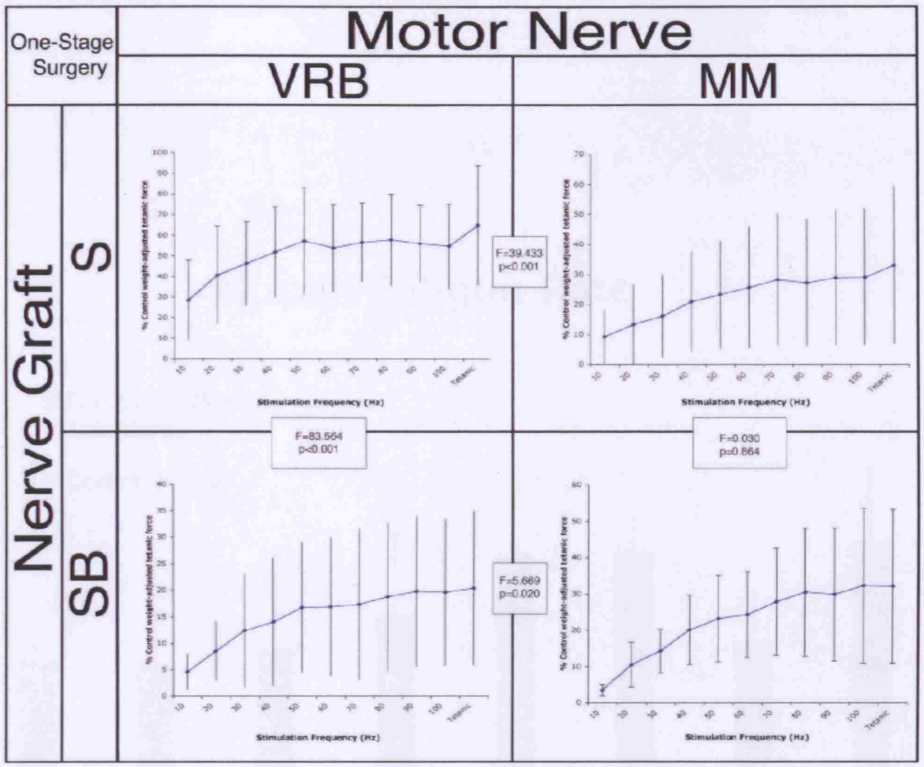



**Figure 5.7.** Mean control-adjusted weight-adjusted force/stimulation frequency relationships for one-stage FMTs. Points represent mean ± SD. Statistical comparisons made using 2-way ANOVA.

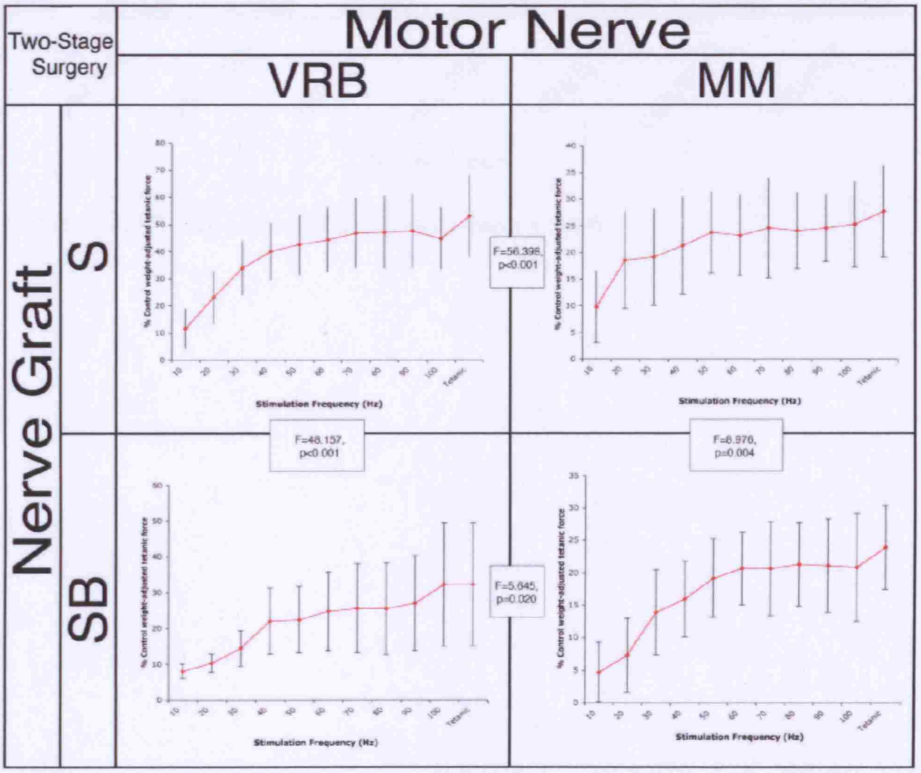



**Figure 5.8.** Mean control-adjusted weight-adjusted force/stimulation frequency relationships for two-stage FMTs. Points represent mean ± SD. Statistical comparisons made using 2-way ANOVA

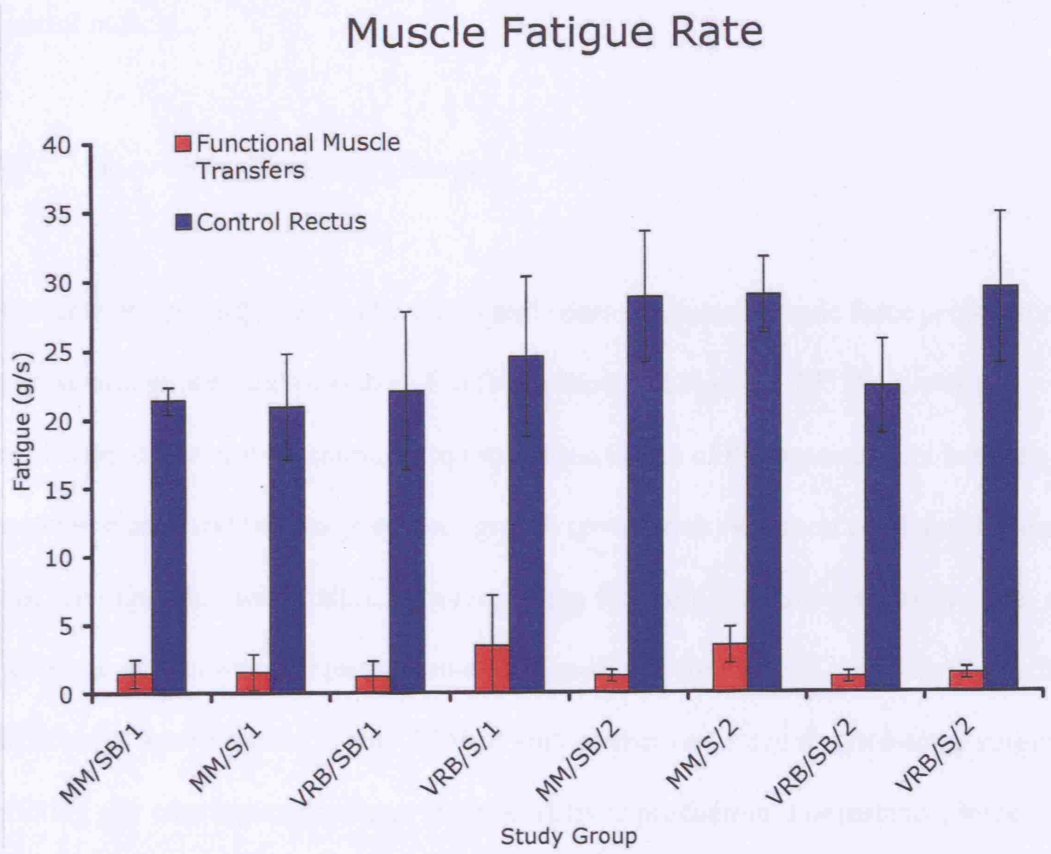



**Figure 5.9.** Fatigue rates for FMTs versus control rectus femoris (Mean $\pm$ SEM).

1.00). Thus all FMTs and all control muscles exhibited statistically similar within-group muscle fatigue rates, with FMT fatigue rates being significantly lower than those of control muscle.

## 5.9. One- versus Two-Stage Surgery

Absolute, weight-adjusted, and weight- and control-adjusted tetanic force productions by corresponding one- and two-stage FMTs are shown in Figure 5.10. There was no significant difference in tetanic force production in any of these parameters between respective one- and two-stage surgery groups ($p=0.145$-$0.946$, t-test or Mann-Whitney test, see Figure 6.2 for details). However, when force production by respective one- and two-stage groups was compared across all stimulation frequencies, occasional significant differences were exposed (Table 5.1) but with neither one-stage nor two-stage surgery offering any consistent advantage in terms of force production. For instance, force production across all stimulation frequencies for MMS1 and MMS2 were significantly different, favouring the latter, whereas those for VRBS1 and VRBS2 were significantly different, favouring the former. These occasional differences between groups in the context of statistically similar tetanic force production between all group comparisons indicate that, whilst the choice of one- or two-stage surgery may have produced unpredictable differences in the force/frequency relationship of FMTs, it did not influence their maximal tetanic force.

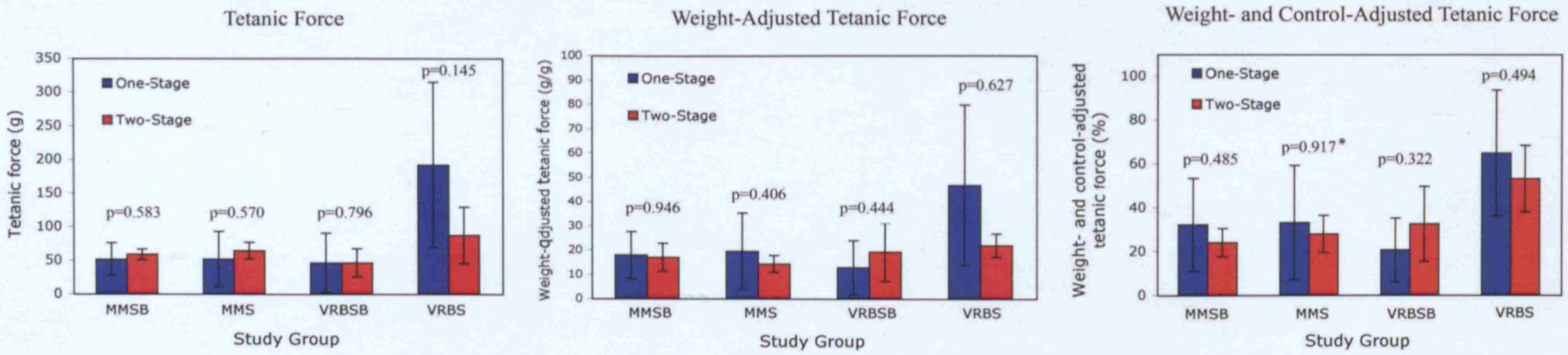



**Figure 5.10.** Absolute, weight-adjusted, and weight- and control-adjusted tetanic force production by FMTs from one- and two-stage groups (mean ± SD). Tetanic force compared between groups using independent samples t-test or Mann-Whitney test (*) if data failed Levene's test for equality of variance.

**Table 5.1.** Comparison of absolute, weight-adjusted, and weight- and control-adjusted force production across all stimulation frequencies by corresponding one- and two-stage FMTs (two-way ANOVA). Statistically significant differences are shown favouring one-(*) or two-stage(†) surgery.

| One- vs. two-stage comparison | Absolute force | | Weight-adjusted force | | Control- and weight-adjusted force | |
|---|---|---|---|---|---|---|
| | F= | p= | F= | p= | F= | p= |
| MMSB | 0.414 | 0.522 | 0.348 | 0.557 | 4.582 | 0.035* |
| MMS | 8.002 | 0.006† | 16.060 | <0.001* | 0.055 | 0.816 |
| VRBSB | 1.239 | 0.269 | 0.001 | 0.973 | 6.401 | 0.013† |
| VRBS | 31.621 | <0.001* | 26.796 | <0.001 | 9.936 | 0.002* |

## 5.10. Discussion

The overall aim of this project was to determine whether it might be possible to achieve nerve graft-dependent, and motor input-independent, FMT neurotisation by use of a low capacity nerve graft. This hypothesis is supported by nerve morphometry data (Chapter 4) but to have any possibility of clinical relevance, FMT pedicle reinnervation must correspond to ultimate force production (and, thence, to clinical outcome). The aim of this experiment was therefore to determine whether different sized motor nerve inputs combined with different capacity nerve grafts affected force production by FMTs in a manner corresponding to the effect seen with neural pedicle reinnervation.

In-vivo measurement of muscle force production poses certain methodological difficulties that had to be considered in order to collect data of the highest possible quality. For instance, whilst muscle force production is known to be dependent on its resting tension (Frey et al., 1983a), physiological resting tension is disrupted by the act of connecting a muscle to a force transducer. Attempts were made to measure force at physiological resting tension by tacking a stretched length of suture to muscle belly before tenotomy, and reattachment to the force transducer at a resting tension which brought the suture fully back out to length. In practice, however, this was difficult to achieve, and fine-tuning of both control muscle and FMT resting tension was performed in all cases by determining the tension producing greatest spike contraction to supramaximal electrical stimulation. Other potential sources of variability were the effects of changes in temperature and muscle desiccation on performance, and muscle

fatigue for later force measurements. These effects were minimised by taking steps to keep the muscle moist by regular irrigation with warmed saline, and minimising the number of electrical stimuli delivered. Nonetheless, these difficulties in measuring force production by muscles in vivo may have introduced inaccuracy.

In addition to absolute force generation data by FMTs ($F_A$), force production was compared in terms of weight-adjusted force-production ($F_W$) and control-adjusted weight-adjusted force production ($F_{CW}$). $F_W$ results were compared to obviate any effect of mass differences between FMTs, and $F_{CW}$ compared to correct, in addition, demonstrable differences in force production by control unoperated rectus femoris muscles which may have signified global differences in strength between animals.

Results in the current study were similar to those obtained elsewhere: for instance, 67.8% control-adjusted weight-adjusted force production has been previously reported in the rabbit rectus femoris FMT model reinnervated without an interposed nerve graft (Frey 1983), compared with 64.7% in this study using an interposed nerve graft (in the VRBS1 group).

In general the results obtained were pleasingly consistent with the hypothesis that force production by muscle may correlate with motor input when a high capacity interposed nerve graft, but not when a small diameter interposed nerve graft, was used. Thus, for both one- and two-stage surgery groups, $F_A$, $F_W$ and $F_{CW}$ of VRBS FMTs significantly exceeded those of MMS FMTs. Conversely when the SB nerve graft was used, the effect

of using large or small motor inputs on these outcomes was greatly reduced, there being either no significant difference between respective one- and two-stage VRBSB vs MMSB groups ($F_A$ of VRBSB1=MMSB1, $F_W$ of VRBSB1=MMSB1, $F_W$ of VRBSB2=MMSB2) or any difference being much attenuated and inconsistently favouring VRB or MM. Overall one may conclude that use of a low capacity interposed nerve graft reduced the high variability caused by differences in motor input magnitude that were evident in the presence of a high capacity interposed nerve graft.

Considering the effect of changing the nerve graft in the presence of a constant motor nerve, the low capacity nerve graft consistently reduced $F_A$, $F_W$ and $F_{CW}$ produced by FMTs reinnervated using the VRB motor nerve. The effect of the low capacity nerve graft on FMTs reinnervated by MM on the same outcomes was less consistent, there being either no significant difference between MMS and MMSB or the former slightly exceeding the latter. These observations are consistent with the interpretation that axon sprouts produced by the transected MM could, in general, be fully accommodated by S nerve grafts and just about accomodated by SB nerve grafts, there therefore being little or no difference between MMS and MMSB FMTs (differences occuring as a result of slight variability in the axon content of individual motor nerves, properties of the nerve grafts, or random chance); whereas axon sprouts produced by the transected VRB were accomodated to a much leser extent by SB than S nerve grafts, there therefore being consistently superior force production by FMTs reinnervated via the latter nerve graft when VRB was the reinnervating motor nerve.

Transmission fatigue is the rate of decline of isometric tension developed by a muscle (Thomson, 1950). In the current study, there was no significant difference between fatigue rate for any two FMT or control muscle groups. However, control muscles overall exhibited much more rapid fatigue than FMTs.

Absolute, weight-adjusted, and weight- and control-adjusted tetanic force productions by corresponding one- and two-stage FMTs were statistically similar, although occasional differences were found on more in-depth comparison of force production across all stimulation frequencies. These occasional differences between one- and two-stage surgery groups favoured neither consistently and, in the context of statistically similar tetanic force production, indicated that the choice of one- or two-stage surgery could produce unpredictable differences in the force/frequency relationship of FMTs but did not influence their maximal tetanic force. Overall the results from this section have failed to identify any advantage of two-stage over one-stage surgery.

# CHAPTER 6

# CONCLUSIONS

## 6.1. Conclusions

The current series of results was examined as a whole to assess whether overall the study hypothesis should be supported or rejected.

### 6.1.1. Study Hypothesis

FMT combined with CFNG is the facial reanimation procedure of choice for chronic one-sided facial palsy but is limited by its unpredictable outcome (Gary-Bobo et al., 1980; O'Brien et al., 1990; Terzis and Noah, 1997; Chuang, 2002; Kumar and Hassan, 2002; Hamilton, 2003; Yla-Kotola et al., 2004). This may result from variations in motor input arising, for instance, from variable facial nerve anatomy (Kwak et al., 2004) and random axon loss at each coaptation and across the interposed nerve graft. This notion is supported by previous work indicating that variable motor input correlated with reinnervation and force production in an experimental FMT model (MacQuillan, 2005) and the view that FMTs for facial reanimation are highly sensitive to small changes in reinnervating load resulting from a low axon:muscle fibre ratio and a concomitant tendency to form maximally-sized motor units (Urso-Baiarda et al., 2006). The corollary is that it may be possible to reduce outcome variability by limiting FMT neurotisation in a predictable manner.

Nerve grafts vary in the degree to which they are supportive of axon regeneration, based on factors including physical characteristics and Schwann cell content (termed high and

low capacity nerve grafts in this work). It was hypothesised that a high capacity interposed nerve graft permitted high fidelity transmission of axons from motor nerve to periphery and thereby supported variability in final outcome, whereas a low-capacity nerve graft limited neurotisation both of the itself and of any coapted end organ in a nerve graft-dependent manner. Thus a low-capacity interposed nerve graft could limit axon regeneration across it between donor nerve and FMT, limiting FMT reinnervation and producing more consistent force production in the face of inconsistent donor nerve input.

The principle aim of this study was therefore to test the hypothesis that it might be possible to limit FMT reinnervation in order to produce more predictable force production despite variable motor input by means of properties of the interposed nerve graft. A related study aim concerned with optimising facial reanimation surgical technique for arose from the need for data collection using both one- and two-stage surgery methodologies. Both were investigated since each is practised clinically, neither being universally accepted as gold-standard, and it was felt that data from one could not reliably be applied to the other due to differences in end-organ neurotrophic effects and FMT denervation atrophy. Consequently, each one-stage group had a matched two-stage group, permitting comparison of the outcome of one- and two-stage surgery across a range of reinnervation conditions.

### 6.1.2. Nerve Morphometry

The principle aims of nerve morphometry experiments were: at the 0 month time point, to confirm the selection of two motor nerve branches and two nerve grafts containing significantly different myelinated fibre contents (and, in the case of nerve grafts, cross sectional areas) from each other, representing different motor inputs and different capacity nerve grafts repsectively; and at 3 and 9 month time points, to compare nerve graft and rectus nerve reinnervation across study groups. Regarding the first of these aims, $VRB_0$ nerve biopsies contained over 7 times more axons than $MM_0$ biopsies, axons being similarly sized in each. $S_0$ contained approximately 2.5 times more axons than $SB_0$, which were also significantly larger than the latter. $S_0$ nerves were on average x times larger than $SB_0$.

Morphometry of distal nerve graft biopsies in two-stage groups at 3 months revealed that axon size was the same in all study groups (3-4$\mu m^2$), and considerably less than in both original motor nerves and original nerve grafts (18-19$\mu m^2$ and 6-8$\mu m^2$ respectively) Similarly reduced diameter axon regenerates within a nerve graft have been shown previously in the rat sciatic nerve (Ansselin and Davey, 1988) and are consistent with the formation of slender axon sprouts as an early event in nerve regeneration (Morris et al., 1972; Kim et al., 1990). Three-month nerve neurotisation of the high capacity S interposed nerve graft[9] was greater for the larger VRB than for the MM motor nerve. However, three-month neurotisation of the low capacity SB graft was similar irrespective of the motor nerve used. These observations were consistent with there being an upper

[9] as determined by axon number

limit to axon transmission by the SB nerve graft that was achieved by both motor nerves. Neurotisation therefore became graft-dependent and motor nerve-independent.

Conversely, maximal motor unit expansion by the MM motor nerve was insufficient to neurotise the S nerve graft maximally, graft neurotisation therefore being limited only by the capacity of that nerve for axon sprouting. Axon branching by MM into the S nerve graft was 320% of the original MM count, pleasingly consistent with earlier accounts of maximal facial nerve motor unit expansion by a factor of 2-3 (Frey, 1998).

In contrast to use of the SB interposed nerve graft, S graft neurotisation by VRB exceeded that of MM, since the larger VRB motor nerve was capable of expansion into more of the Schwann tubes left vacant despite maximal MM expansion. Expressed as a percentage of original axon count, however, MM exceeded 300% expansion whereas VRB achieved only 64% of its original axon count within the S nerve graft. Whilst this result could have been attributed to intrinsically inferior axon sprouting by VRB than by MM, previous studies using reversed sections of VRB as autologous nerve grafts for repair of segmental VRB defects found similar axon counts in both VRB and nerve graft (Spector, 1997). The more likely explanation is therefore that maximal transmission of VRB sprouts had been impaired even by the largest S interposed nerve graft.

Nine-month nerve graft neurotisation was compared on the basis of total axon area as inconsistent biopsy sampling location may have confounded number:area comparisons (discussed in Chapter 4 Section 4.8.3). This was not the case for nine-month rectus

neurotisation, which could therefore be compared on the basis of axon number and total axon area (there being no difference between groups in mean axon area). Consistent with three-month data, and for both one- and two-stage study groups, neurotisation of either the nerve graft or the rectus nerve was superior by a reinnervating VRB motor nerve compared with MM only when a high capacity S interposed nerve graft was used, there being no difference between them when the SB interposed nerve graft was used.

Overall, therefore, nerve morphometry data was consistent with the hypothesis that FMT reinnervation could be limited and made more predictable by means of the interposed nerve graft despite variable motor input. Where motor input exceeded nerve graft capacity, axon loss was presumed to be in the form of neuromas at the points of neurorrhaphy in keeping with previous reported observations (for instance, that increasing suture line neuromas occurred in a rat sciatic nerve repair model using 100%, 250% or 350% of the original axon input (Mackinnon et al., 1989)).

### 6.1.3. Physiology

Comparison of muscle physiology data was based on control-adjusted values since significant differences were detected amongst some control group comparisons. Previous studies have also expressed FMT force production (reinnervated without a nerve graft) as a proportion of that of control, with similar results to those presented here when the additional influence of an interposed nerve graft is considered. For instance, Guelinckx (Guelinckx et al., 1984) achieved 32% of control force production by orthotopic FMTs

reinnervated without an interposed nerve graft in the rabbit rectus femoris model, compared with 26% for FMTs from the VRBS1 group in the present study, via an interposed nerve graft.

Control-adjusted force production by one- and two-stage FMTs correlated with motor input magnitude for the larger S nerve graft but, at least for one-stage surgery groups, not for the SB interposed nerve graft. There was one inconsistent result in that weight-adjusted force production of two-stage FMTs reinnervated via the SB nerve graft exhibited significantly greater force when reinnervated by VRB than by MM, although this difference was greatly reduced compared with that of similar groups reinnervated via the S nerve graft (Chapter 5, Section 5.6.2) (there being a 9% difference in control-adjusted force production between motor nerves reinnervated via S, compared with a 2% difference between those reinnervated via SB). Although two-stage FMTs reinnervated via SB and either the MM or VRB motor nerve did not exhibit statistically indistinguishable control-adjusted force, a standardising effect of the low capacity graft nonetheless appeared to be present.

Muscle physiology data therefore generally also supported the hypothesis that force production by FMTs reinnervated via a high capacity interposed nerve graft correlates with motor input magnitude, but that this correlation is absent when a low capacity interposed nerve graft is used.

### 6.1.4. One- versus Two-Stage Surgery

A secondary aim of this study was to compare outcome in terms of neural reinnervation and force production by matched groups that underwent either one- or two-stage surgery. Despite published experimental evidence suggesting that one-stage FMT combined with CFNG may be equally or more efficacious than two-stage surgery, the traditional surgical preference for two-stage surgery persists. Mackinnon and colleagues demonstrated superior nerve regeneration[10] across sural nerve grafts coapted proximally to a facial nerve branch in monkeys with, compared to without, distal attachment to facial muscle, but concluded for clinical practice that the distal nerve graft should be sutured to the distal facial nerve trunk on the paralysed side before performing FMT as part of a two-stage procedure (Mackinnon et al., 1988), although the neurotrophic and neurotropic effectiveness of chronically denervated, dysfunctional atrophic facial muscle is open to debate.

Similarly, despite showing that one-stage FMT with CFNG resulted in similar FMT tetanic force production and superior neural pedicle reinnervation compared with two-stage surgery, Rab and colleagues continued to favour two-stage surgery based on reduced versatility and the need to create additional operative scars in one-stage surgery (Rab et al., 2006). This is based on the historical practice of surgeons to perform one-stage facial reanimation using a FMT with a long neural pedicle in lieu of a nerve graft, in the belief that axons will not successfully cross a second coaptation if FMT and CFNG

[10] As indicated by mean axon areas of 3.08 $\mu m^2$ vs. 2.37$\mu m^2$, with fewer epineurial regenerating units.

are performed during the same operative procedure (Anderl, 1973). This limits the choice of FMT (latissimus dorsi being the commonest choice), the FMT's range is reduced and an additional scar is needed to tunnel the neural pedicle.

In keeping with this tradition, the experimental model used by Rab et al. compared two-stage surgery using a FMT and an interposed nerve graft, with one-stage surgery using a FMT with a long neural pedicle (and no nerve graft). Superior reinnervation outcomes were ascribed to the avoidance of a distal coaptation (Rab et al., 2006). In this respect the current experimental model, in which a nerve graft was used for both one- and two-stage procedures, offered the advantage of fair one- versus two-stage comparison without differences in the presence or absence of a distal coaptation. Additionally, if shown to produce satisfactory outcomes in an experimental setting, the clinical use of one-stage FMT with nerve grafting would obviate the disadvantages cited by Rab and colleagues of an additional scar and reduced versatility (Rab et al., 2006).

In the present work, nerve morphometry and muscle force production data failed to shown any significant benefit from performing two-stage CFNG followed by FMT, compared with one-stage surgery. One-stage surgery has advantages including fewer operative procedures and therefore decreased operative risk, shorter total recovery time (Harii et al., 1998; Kumar and Hassan, 2002) and greater efficiency in the use of healthcare resources. Opponents criticise one-stage surgery based on an increased period of deleterious denervation atrophy (Richter et al., 1979; Fu and Gordon, 1995; Aydin et al., 2004), reduced versatility and the need to create additional operative scars in one-

stage surgery (Rab et al., 2006). Of these criticisms, the first is unsupported by the current findings, and the latter two obviated by performing one-stage surgery using a nerve graft, exactly as is done for two-stage surgery.

A limitation mandated by the study design was the use of a 3cm length of interposed nerve graft, there being available no paired sensory nerves of greater length and of dissimilar axon number and/or area, as required to fulfill the main research aims of this project. This may impair the applicability of these results to humans, in whom 15-20cm interposed nerve graft lengths are usual with a concomitant increased FMT denervation period related to axon regeneration across a nerve graft for one-stage procedures.

Nevertheless, the corroboration of previous experimental research utilising longer graft lengths, combined with the absence even of a statistically insignificant trend towards better outcomes of two-stage surgery in the current work, and the clinical importance of the issue of superiority of one- or two-stage surgery, warrants future investigation specifically directed at FMT recovery following either one-or two-stage surgery using interposed nerve grafts in each case.

## 6.2. Overall Conclusions

In conclusion, the series of experiments presented in this thesis support the hypothesis that the use of a nerve graft which limits axon transmission between motor nerve and

FMT may standardise reinnervation and force production by the latter despite variability in motor input.

Results from this work do not support the view that two-stage surgery offers any advantage in terms of outcome compared with one-stage surgery, nor that it is necessary to avoid the use of an interposed nerve graft in one-stage surgery.

Other conclusions that may be drawn from this series of experiments are: the confirmation of previous studies showing that marginal mandibular and ventral ramus of buccal branches of the facial nerve can be used in the rabbit rectus femoris FMT model to provide different reinnervating motor inputs (MacQuillan, 2005); that the medial saphenous nerve (not previously described in the literature) may successfully be used as a nerve graft for muscle reinnervation, and that its axon transmission capability differs sufficiently from that of the saphenous nerve to enable their experimental comparison. This study also represents the first body of work to utilise the nerve morphometry quantification method described in Chapter 3. The similarity of results with previous data obtained using more time-consuming methods (Chapter 4 Section 4.8.4) supports its efficacy.

## 6.3. Axon Transmission Capacity by Interposed Nerve Grafts: Mechanism of Action

The principle aim of this study was to determine whether properties of the interposed nerve graft could be used to standardise innervation and force production by a FMT, but there was no attempt to prove how the nerve graft may do this. S and SB nerve grafts differed both in their myelinated fibre content at the time of harvest and in their physical diameters, in both respects S exceeding SB, and either, both or additional unrecognised factors could have contributed to SB nerve grafts being less permissive to axon transmission.

It has previously been shown that Schwann cells survive within nerve grafts (Richardson et al., 1983) and both enhance (Guenard et al., 1992) and direct (Riopelle et al., 1981) neural regenerates towards end organs, acting as physical conduits via the formation of Schwann tubes (Scherer and Easter, 1984) and as the sources of trophic and tropic neural factors (Daniloff et al., 1986). In this study, S may have exhibited superior axon transmission capability compared with SB nerve grafts as a result of containing more Schwann cells and/or Schwann tubes. This may be suspected by the presence of more myelinated nerve fibres at the time of graft harvest in S compared with SB, but involves two assumptions: firstly, that myelinated fibre number at the outset of experiments correlated with Schwann cell number; and secondly, that the latter correlated with Schwann cell numbers at the end of experiments. The first assumption is based on the fact that Schwann cells form an axon's myelin sheath by spiralling round it, and Schwann cell number must therefore be related to myelinated fibre number. However, the relationship also depends on myelin thickness, which was not measured in this study.

The second assumption - that Schwann cell numbers at the time of graft harvest correlated with those at the final experiments - is not assured since it is known that Schwann cell proliferation takes place after nerve injury (including nerve graft harvest). Whilst there is no reason to suppose different rates of Schwann cell proliferation in S and SB nerve grafts, such a difference is possible (for instance resulting from different graft thicknesses and oxygen penetration during early graft take (Best et al., 1999)) and early differences between grafts in initial Schwann cell number could have been lost as a result. Whilst a difference in axon transmitting capability between S and SB nerve grafts has therefore been shown, its dependence on Schwann cell content has not been proven. Moreover, the existence of axon regeneration across nerve grafts that was independent of Schwann cells of nerve graft origin is suggested by observations of a minority of axon regenerates clumped into 'mini-fascicles' (Figure 7.1). These have been previously observed in very wide diameter nerve grafts prone to ischaemia (Schroder and Seiffert, 1970; Millesi, 1984) and were the sole type of axon regenerates across acellular nerve grafts (Hall, 1986), suggesting they might characterise axon regeneration utilising Schwann cells derived from the motor nerve rather than from the nerve graft.

An alternative, and not mutually exclusive, cause of differential axon transmission capability of S and SB nerve grafts may have been their different physical diameters, limiting either the physical capability for axon transmission directly (by exerting an upper

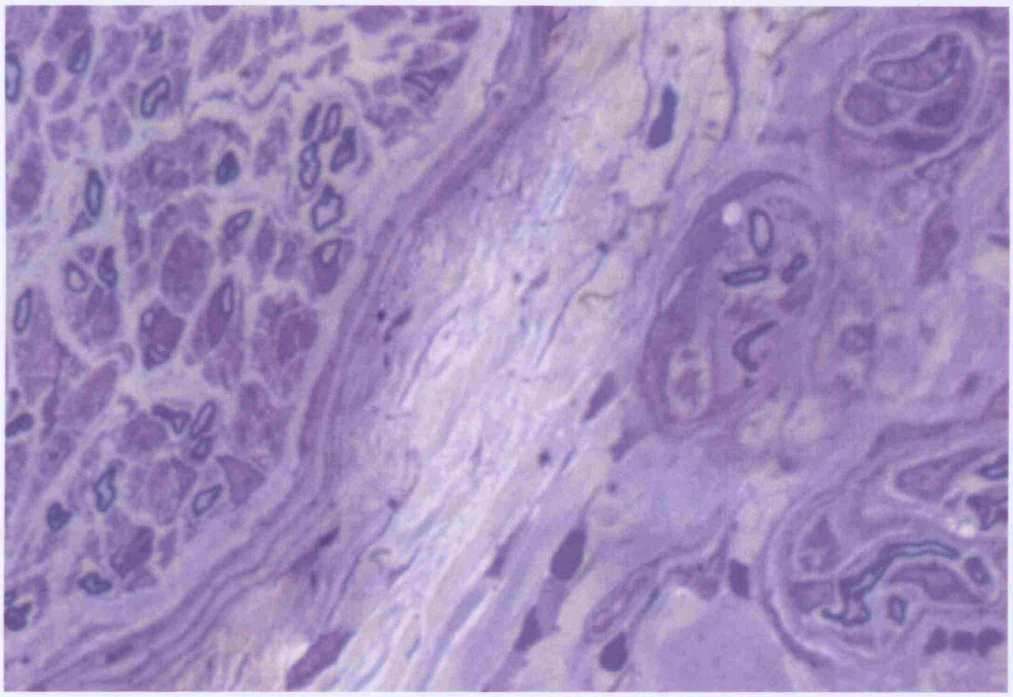


**Figure 6.1.** An example of mini-fascicle formation within nerve grafts at three months.

limit on the total axon cross sectional area that could be packed into the nerve graft) or doing so indirectly, for instance by limiting the potential for Schwann cell proliferation. Although total axon area within nerve grafts never approached 100%, even in the smallest SB grafts neurotised by the largest VRB motor inputs, this does not preclude the possibility of such physical limitation on total axon regenerates, since it is likely that much of the nerve graft area not occupied by regenerating axons was composed of Schwann cells, blood vessels and supporting parenchyma essential for nerve graft integrity, that was not available for occupation by axon regenerates.

## 6.4. Implications for Facial Reanimation Surgery

This work aimed to identify a surgical strategy for standardising the outcome of FMT combined with CNG that could be applied to facial reanimation in humans. This section addresses how this research could be put to use in a clinical setting, and potential objections to limiting axon transmission by use of the interposed CFNG that may oppose its practice.

Clinical outcomes of FMT with CFNG range from underactive to overactive, whereas limiting FMT reinnervation intuitively improves only overactivity. In this respect, standardising reinnervation using a CFNG represents only part of a successful clinical strategy, the other part being the supply of a donor motor input that is sufficiently in excess of the transmission capability by the interposed nerve graft so as to remove the

effects of random losses at the proximal coaptation and across the nerve graft itself, which may previously have contributed to FMT underactivity.

Related to this objection, surgeons traditionally aim to optimise FMT reinnervation through meticulous tissue handling and coaptation technique. The concept of sabotaging maximal axon delivery through use of a low capacity interposed nerve graft may therefore be unappealing. However, it should be appreciated that this strategy is not intended to prevent optimal reinnervation being achieved by FMTs but to avoid the fluctuations in neurotisation accommodated by a large capacity nerve graft. The objective is to guarantee sufficient reinnervation by use of significant redundancy of motor nerve input, and then to prevent excessive reinnervation by the limiting influence of the interposed nerve graft, such that at the distal end of the nerve graft a constant and predictable motor nerve 'dose' is delivered to FMTs in each case. Whilst there remains some scope for downstream variation in the axon load actually reaching muscle (principally through loss at the distal coaptation), this is considerably reduced compared with the usual cumulative sources of variability to that point.

With regards to the requirement for a large redundant motor input, potential objections centre on increasing donor morbidity (in the form of excessive weakness of part of the normal hemiface supplied by the nerve used to reinnervate the contralateral FMT) and the need to select a functionally correct motor nerve branch for the transplanted muscle. However, it has been remarked previously that donor morbidity is usually minimal, due to extensive ramifications between nearby branches, and aproblematic, since a degree of

weakness actually improves symmetry. Options for selecting the functionally correct motor nerve branch whilst maintaining motor redundancy include coapting the nerve graft more proximally than normal, perhaps in an end-to-side manner to prevent marked donor morbidity (Cederna et al., 2001), or augmenting the innervation provided by the functionally optimal nerve with one or more neighbouring nerves.

One further uncertainty relating to the low capacity nerve graft strategy involves the need to balance predictability of outcome with impairment of function, and the question of exactly how low in capacity the nerve graft should optimally be. A very low capacity nerve graft has a high probability of transmitting axons up to that capacity irrespective of the magnitude of motor input, with therefore high predictability of axon delivery at the expense of high attenuation in axons reaching the end organ. As nerve graft capacity increases so does the possibility of not meeting it. Thus outcome predictability versus functional impairment may be thought of as lying along a spectrum, ranging from the acellular long nerve graft (lowest capacity) which produces an extremely consistent outcome, but consistently very poor (Hall, 1986), to a high capacity, Schwann cell rich, wide diameter nerve graft (more analogous to the current clinical situation) which optimises force production by the functional muscle transfer at the expense of high outcome variability.

In the present study, use of the low capacity SB nerve graft produced more consistent results than the S nerve graft, but force production was much impaired (around 10% control-adjusted force, versus up to 26% using the larger nerve graft). Importantly

however, force production was quoted as a percentage of rectus femoris control, although it is more relevant to understand force production in terms of contralateral mimetic musculature. It is possible that 10% of rectus femoris function represented a satisfactory reconstruction for mimetic musculature. Experimental FMT models permitting force measurement of mimetic musculature, such as the rabbit scutuloauricularis model (Nehrer-Tairych et al., 2000), could be used to investigate the relationship between axon diameter and predictability of outcome with a view to determining an optimal balance.

Implicit in the hypothesis that a narrow diameter, Schwann cell poor nerve graft could produce nerve graft-dependent end organ reinnervation that is more predictable than the alternative of motor nerve-dependent reinnervation is the expectation that physiological variability of the nerve graft is less than that of the facial nerve. This is yet to be demonstrated in a human anatomical study, although one supposes it unlikely that the particular variability of facial nerve ramification (Kwak et al., 2004) would be matched by all available nerve graft options. However, research investigating the variability of nerve morphometry analyses of commonly used human facial nerve branches and nerve grafts could usefully address this assumption.

Certain differences between the work presented here and the clinical situation preclude direct extrapolation of results and conclusions from one to the other, the most obvious being the use of an animal model. In many cases, attempts were made to ensure that similarities to clinical practice were maintained as closely as possible: for instance, by performing heterotopic muscle transfer and reinnervating it using facial nerve branches.

In other instances, differences were deliberately introduced to address questions left unanswered by previous research: thus, one-stage surgery utilised a nerve graft rather than a long motor nerve pedicle, as performed by Rab and colleagues (Rab et al., 2006) and discussed earlier. Finally, some differences were unavoidable due to the constraints imposed by the animal model used. These included: the selection of FMT, rectus femoris, not being the preferred muscle of choice for human facial reanimation; the use of an ipsilateral nerve graft rather than a cross-facial one (discussed in Chapter 2, Section 2.4.2b); and differences in nerve regeneration, including across nerve grafts, between animals and humans.

One significant difference from human facial reanimation mandated by the study design was the use of a 3cm length of interposed nerve graft, there being available no paired sensory nerves of greater length and of dissimilar axon number and/or area, as required to fulfill the main research aims of this project. Whilst not problematic for evaluation of the effects of the interposed nerve graft on motor input-induced FMT variability, this could impair the applicability of one- versus two-stage data to humans, in whom 15-20cm interposed nerve graft lengths are usual with a concomitant lengthier FMT denervation period related to axon regeneration in one-stage procedures. Nevertheless, the corroboration of previous experimental research finding similar one- versus two-stage outcomes utilising longer (7cm) nerve lengths (Rab et al., 2006), combined with the absence even of a statistically insignificant trend towards better outcomes of two-stage surgery in the current work, and the clinical importance of the issue of superiority of one-

or two-stage surgery, warrants future investigation specifically directed at FMT recovery following either one-or two-stage surgery using interposed nerve grafts in each case.

Finally, experimental groups in this research work were reinnervated using entirely different facial nerve branches consistently exhibiting large differences in axon content, to simulate in humans smaller differences in axon load provision occurring by chance when using similar facial nerve branches. In the clinical setting, more modest differences in reinnervating axon load are expected between cases than those imposed experimentally in this study. The success of the strategy of limiting axon transmission by use of a low capacity interposed nerve graft in standardising FMT outcome under these more rigorous experimental conditions therefore holds promise for its potential usefulness as a strategy for human facial reanimation surgery.

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